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Rhinovirus detection using probes from the 5′ and 3′ end of the genome

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Summary. This study investigated the abilities of cDNA probes from the 5′ and 3′ ends of the genome of human rhinoviruses (HRV-) 14, 9, and 1B to detect RNA from 59 rhinovirus serotypes. The results show that probes from the 5′ end of the genomes of HRV-14, 9, and 1B detected a large number of serotypes but the detection rate was variable and depended on the degree of homology with the particular probe. In contrast, all the 3′ end probes were specific for the homologous virus. However, a *long* HRV-9 probe detected a large number of serotypes.

It was concluded that such cDNA probes would not detect all serotypes with equal efficiency. Synthetic oligonucleotides corresponding to short but highly conserved regions in the 5′ non coding region may overcome this problem.

Introduction

Rhinoviruses are the major causative agents of the common cold [9]. In the majority of *healthy* individuals, the infection results in a short illness of some 3–5 days duration characterized by rhinorrhoea, nasal obstruction, sore throat and pharyngitis [4]. However, in immunocompromised individuals particularly children and in patients with obstructive airways disease, rhinovirus infection may result in more serious lower respiratory tract involvement [11, 14]. Furthermore, recent community studies in Michigan, U.S.A., suggested that rhinoviruses can be isolated from up to 70 percent of adults (over 40 years of age) with lower respiratory tract involvement [15]. In these individuals the median duration of illness was as long as 3 weeks [15].

We have recently shown that a new synthetic anti-rhinovirus agent, R61837, can sucessfully suppress illness in volunteers challenged with a rhinovirus [5]. It is anticipated that with further progress in the field it may be possible to

treat these infections. However, rapid virus identification prior to treatment would be essential since these antivirals are specific for rhinoviruses. Until recently, rhinoviruses could only be identified by growth in a sensitive cell culture. Such procedures are time consuming, labour intensive and require considerable expertise. Although, it is now possible to detect rhinovirus antigens directly in nasal washings using immunologically based methods such as ELISA [10], the diversity of serotypes, recently estimated to be around 100 [12] makes efficient detection of all serotypes difficult. We have therefore, attempted to overcome this problem by developing procedures based on RNA detection.

A previous study has shown that a cDNA probe from the 5′ non-coding region of HRV-14 detects 96.4% of the 54 rhinoviruses investigated, but the sensitivity of detection was variable and presumably depended on the degree of genomic homology of particular serotypes with HRV-14 [2]. In this study we therefore increased the number of probes used to include the 3′ ends of HRV-14, 1B and 9 and the 5′ ends of HRV-1B [13] and 9 [Leckie et al., in prep.]. The aim was to find a probe that would hybridise with RNA from various serotypes with equal efficiency and we therefore studied the reactions of the new probes with RNA from 59 rhinovirus serotypes.

Materials and methods

Stocks of rhinoviruses including animal rhinoviruses such as the calf rhinovirus SDI and bovine rhinovirus EC11 and other control viruses, namely influenza A and B (FLU A and B), coronavirus 229E and coxsackie A21 (COXA21) were prepared as previously described [2]. Each stock was titrated in microtitre plates and titres expressed as $TCID_{50}$/ml. Table 1 shows the final titres of the virus stocks as used in this study.

Viral RNA was extracted using the method of Rotbart et al. [16, 17]. Briefly, 0.2 ml of each virus preparation was mixed with an equal volume of a 3 : 2 mixture of 20 × SSC—37% formaldehyde. The mixture was then spotted onto nitrocellulose filters that had been pre-soaked in 20 × SSC as described earlier [2].

Details of the methods used to prepare and label probes used in this study have been described previously [2, 3]. Briefly, probes were produced from M13 templates containing rhinovirus cDNA cloned in the appropriate orientation. The HRV-14 5′ construct contained nucleotides 1-802 and was produced by cutting a recombinant plasmid with *Pst*I and *Bgl*II and ligating into the M13 mp19 *Pst*I and *Bam*HI sites. The corresponding 3′ probe comprised positions 6336-7167 contained within an *Hpa*I fragment ligated into M13 cut with *Sma*I. The HRV-1B probes represented positions 1-846 (5′) located within a *Pst*I *Hin*dIII fragment which was ligated into these sites of M13 and 6338-7133 (3′) located in a *Pst*I fragment.

The HRV-9 5′ end probe was prepared from a 3.3 kb cDNA clone of HRV-9 designated pR9112. A 472 base pair fragment representing nucleotides 1-472 of the HRV-9 sequence (unpublished) was subcloned into M13 mp18 in the positive sense orientation. Both of the 3′ end probes used in this study were prepared from a 1.1 kb HRV-9 cDNA clone pR9193 covering the 3′ region of the genome. The *short* 3′ end probe was prepared from a 331 base pair fragment representing nucleotides 6768-7098. The total length of the HRV-9 genome is 7128 excluding poly A tail. The *long* 3′ end probe was prepared from a 769 base pair fragment from pR9193 covering nucleotides 6005-6773. In both cases the fragments were subcloned into the phage vector M13 mp18 in the positive sense orientation. The templates were used to produce radioactive cDNA probes complementary to viral sense

RNA by extension of an M13 universal primer in a reaction performed by the Klenow fragment of DNA polymerase 1. Annealing of the primer/template was achieved by boiling together (3 min) in a mixture (20 µl) comprising primer (5 ng), template (1 µg), 15 mM Tris-HCl, pH 8.0, and 7.5 mM $MgCl_2$. The solution was allowed to cool to room temperature and to it was added dGTP, dCTP dTTP (to a final concentration of 0.5 mM), ^{32}P- dATP (20 µCi) Klenow fragment (5 units) and water to give a final volume of 50 µl. The reaction took place at room temperature for 30 minutes after which the radioactive DNA was separated from unincorporated nucleotides by passage through a Sephadex G100 column.

The probes were hybridized with viral RNA as described previously [2, 3]. The strength of the hybridization signals was assessed visually by two independent observes and the signal was classified as very strong (+ + + +), strong (+ + +), good (+ +), positive (+), weak (±), or no signal (0).

Results

Comparison of the hybridization results with the titres of the virus stocks (Table 1) shows that generally there is no direct relationship between the titres and the strength of the hybridization reaction. For example, the HRV-14 probe hybridised very strongly (+ + + +) with HRV-49 even though the titre was low ($< 10^4$ $TCID_{50}$/ml) but only weakly with HRV-1A (+) which had one of the highest titres of the viruses tested ($> 10^8$ $TCID_{50}$/ml) (Table 2). It therefore appears that the efficiency of detection is more directly related to other factors, the most important of which is probably the degree of RNA homology between the RNA of the different rhinoviruses.

Table 2 shows the strength of the signal observed using the various viruses and seven probes. It can be seen that the reactions varied greatly in intensity. Thus, the HRV-14, 5′ end probe gave a very strong signal (+ + + +) when hybridized with RNA from HRV-3, 4, and 49 and a strong signal (+ + +) when reacted with RNA from HRV-2, 41, 47, 56, 62, 72, and 85. Similarly, a 5′ end probe from HRV-9 gave a very strong signal (+ + + +) with RNA from HRV-15, 31, and 32.

The 5′ end probe from HRV-1B hybridized very strongly (signal + + + +) with RNA from HRV-49 and 85 and strongly (+ + +) with RNA from HRV-1A, 15, and 19.

In contrast to the 5′ end probes, those from the 3′ end (831, 795, and 331 nucleotides in length for HRV-14, 1B, and 9, respectively) detected only the homologous virus in the conditions of the assay. However, the *longer* probe (768 nucleotides in length) from HRV-9, detected many more viruses (Table 2). Indeed, this probe hybridized very strongly (+ + + +) with RNA from HRV-13, 24, 27, 32, 64, 73, and 75 and reacted strongly (signal + + +) with RNA from HRV-11, 15, 18, 41, and 65 suggesting that these viruses are closely related to HRV-9 in the 3′ end region of the genome and perhaps reflects the conservation of the polymerase sequences among these viruses.

Both 5′ and 3′ end probes from HRV-14, -9, and -1B reacted with their respective viruses very strongly (+ + + +).

Table 1. Titres of rhinovirus and control virus stocks

Virus	Titre ($10\,TCID_{50}$/ml)	Virus	Titre ($10\,TCID_{50}$/ml)
Rhinovirus 1A	$\geqslant 8$[a]	Rhinovirus 42	6.5
1B	6.75	43	$\geqslant 8$
2	6.25	44	<4
3	$\geqslant 8$	45	6.5
4	$\geqslant 8$	47	<4
5	5.75	48	4.75
6	$\geqslant 8$	49	<4
7	4.75	51	<4
8	7.0	55	6.0
9	6.5	56	6.25
10	5.25	62	4.5
11	4.75	63	5.75
12	6.75	64	6.5
13	$\geqslant 8$	69	6.5
14	6.25	70	6.0
15	7.25	72	7.0
16	6.25	73	5.5
17	6.75	75	6.5
18	5.5	77	<4
19	5.5	80	6.25
20	6.25	81	5.75
23	4.5	82	5.25
24	6.25	85	6.25
25	4.25	EL	6.75
27	6.0	EC11	6.0
28	6.0	SD1	4.75
29	$\geqslant 8$	Coxsackie A21	7.5
30	$\geqslant 8$	Coronavirus 229E	7.45
31	5.0	Influenza A	7.25
32	$\geqslant 8$	Influenza B	7.0
40	4.75		
41	5.75		

[a] The titre for HRV-1A is $\geqslant 10^8\,TCID_{50}$/ml

Table 3 shows the percentage of rhinovirus serotypes detected by each probe according to the strength of the hybridization signal. Thus 93.2, 66, and 74.5% of viruses investigated were detected (signal $>+$) by HRV-14, 9, and 1B, 5′ end probe, respectively, while 71% were detected by the *long* 3′ end HRV-9 probe. Similarly, 45.8, 20.3, and 37.3% of rhinoviruses gave a good hybridization signal ($>++$) with 5′ end probes from HRV-14, 9, and 1B, respectively, while 35.6% gave a similar signal with the *long* HRV-9, 3′ end probe.

As can be seen from Table 2, none of the control respiratory viruses such as influenza A, B, and coronavirus 229E, gave positive hybridization signals

Table 2. Rhinovirus detection using different cDNA probes

Virus	Probe	Strength of hybridization signal					
		++++	+++	++	+	±	0
HRV-14	5′ end	3, 4, 14, 49	2, 41, 47, 56, 62, 72, 85	1B, 9, 12, 13, 17, 19, 23, 29, 30, 31, 32, 48, 64, 65, 80, EL	1A, 5, 6, 7, 8, 10, 11, 15, 16, 18, 20, 24, 25, 27, 28, 40, 42, 43, 44, 55, 63, 69, 73, 75, 77, 81, COXA21, EC11, SDI	45, 51, 70, 82	229E, FLUA, FLUB
	3′ end	14				81, 82, 85	
HRV-9	5′ end	9	15, 31, 32	10, 16, 19, 25, 30, 43, 73, 85	1A, 1B, 2, 3, 11, 13, 23, 24, 27, 28, 41, 42, 47, 49, 55, 56, 62, 63, 64, 65, 75, 77, 80, 81, EC11, SDI, EL	8, 12, 14, 18, 20, 29, 40, 44, 45, 82	4, 5, 6, 7, 17, 48, 51, 69, 70, 72, 229E, COXA21, FLUA, FLUB
	3′ end (short)	9					
	3′ end (long)	9, 13, 24, 27, 32, 64, 73, 75	11, 15, 18, 41, 65	19, 25, 30, 43, 44, 47, 85, SD1	1A, 1B, 2, 4, 5, 6, 7, 10, 16, 23, 28, 31, 40, 49, 56, 62, 63, 70, 72, 77, EC11	3, 8, 14, 42, 45, 48, 80, 81, 82	12, 17, 20, 29, 51, 55, 69, 229E, COXA21, EL, FLUA, FLUB
HRV-1B	5′ end	1B, 49, 85	1A, 15, 19	4, 9, 13, 16, 18, 31, 32, 41, 43, 47, 62, 63, 64, 73, 75, EC11	2, 3, 6, 7, 8, 10, 23, 24, 25, 27, 28, 30, 40, 44, 55, 56, 65, 77, 80, 81, SD1, EL	5, 11, 12, 14, 17, 20, 45, 82, COXA21	29, 42, 48, 51, 69, 79, 72, 229E, FLUA, FLUB
	3′ end	1B			64, 85	1A, 15, 49	

++++ very strong hybridization signals
+++ strong hybridization signals
++ good hybridization signals
+ positive hybridization signals
± weak hybridization signals
0 no hybridization signals

Table 3. Proportion of rhinoviruses detected by the different cDNA probes according to the strength of the hybridization signal

Virus	Probe	Strength of hybridization signal					
		+ + + +	+ + +	+ +	+	> +	> + +
HRV-14	5′ end	4 (6.8)*	7 (11.9)	16 (27	28 (47.5)	55 (93.2)	27 (45.8)
	3′ end	1 (1.7)	0	0	0		
HRV-9	5′ end	1 (1.7)	3 (5)	8 (13.5)	27 (46)	39 (66)	12 (20.3)
	3′ end (short)	1 (1.7)	0	0	0	1 (1.7)	1 (1.7)
	3′ end (long)	8 (13.5)	5 (8.5)	8 (13.5)	21 (35.6)	42 (71)	21 (35.6)
HRV-1B	5′ end	3 (5)	3 (5)	16 (27)	22 (37.3)	44 (74.5)	22 (37.3)
	3′ end	1 (1.7)	0	0	2 (3.4)	3 (5)	1 (1.7)

+ + + + very strong hybridization signals
+ + + strong hybridization signals
+ + good hybridization signals
+ positive hybridization signals
* percent of total 59 rhinoviruses investigated

in any experiments during this study. Coxsackie A21, gave a positive signal with HRV-14 5′ end probe suggesting some genomic homology with HRV-14.

These hybridization tests were repeated three or more times and the results were shown to be reproducible.

Discussion

The data suggest that cDNA hybridization with different probes show a different relationship between rhinovirus serotypes from that based an other properties. For example, HRV-15 which shares the same cellular receptor as HRV-14 (both are included in the major receptor group) [1, 7] reacted more strongly (signal + + +) with the 5′ end probe from HRV-1B, a serotype in the minor receptor group than with HRV-14 (signal +). Similarly, HRV-2 which shares the same cellular receptor as HRV-1B (both are included in the minor receptor group) [1, 7] reacted more strongly (signal + + +) with the 5′ end probe from HRV-14, a serotype from the major receptor group, than with HRV-1B (signal +). Moreover, this relationship is also different from that based antigenic cross-reactivity [8].

It is interesting to note that RNA from HRV-49 hybridized extremely well (signal + + + +) with both HRV-14 and 1B 5′ end probes suggesting a strong genomic homology between HRV-49 and these two viruses. Similarly, RNA

from HRV-15 reacted very well with the 5′ end probe from both HRV-9 and 1B indicating a close genomic relationship between HRV-15 and these two serotypes in the 5′ end. Furthermore, RNA from HRV-15 and 32 hybridized extremely well with both 5′ and 3′ end probes from HRV-9 implying that these viruses have strong genomic homology with HRV-9 in both ends of the genome. In contrast, RNA from HRV-45, 51, 70, and 82 (signal $< +$) and 8 and 81 (signal $+$ to $\pm$) did not hybridize well with any of the probes investigated probably indicating that these viruses are more divergent from HRV-14, 9, and 1B.

The findings of this study are that probes from the 5′ end of the genome of rhinoviruses detect a large number of rhinoviruses, although the detection rate is variable and apparently depends on the strength of genomic homology among the different serotypes. In contrast, probes from the 3′ end of the genome (of some 800 nucleotides) of HRV-14 and 1B detected only the homologous virus under the hybridization conditions of the assay. However, a similar size probe from the 3′ end of HRV-9 detected many more serotypes. In contrast, a shorter probe (331 nucleotides in length) also from the 3′ end of HRV-9, detected only the homologous virus, thus indicating that the detection rate is highly influenced by probe length. Both 5′ and 3′ end probes detected the homologous viruses with equal efficiency.

It was interesting to note that the 5′ end HRV-14 probe was more efficient than the other probes in detecting a larger number of rhinoviruses. This is somewhat surprising since comparative sequence analysis indicates that HRV-14 is relatively diverse from the majority of rhinoviruses studied. It might therefore be thought that probes from HRV-1B and HRV-9, which are more *typical* rhinoviruses, would prove to give a higher detection rate.

The data presented in this paper are interesting in that they show that cDNA probes are unlikely to be useful in detecting all rhinoviruses with equal efficiency despite an earlier prediction that the 5′ end non-coding region was likely to be relatively highly conserved throughout the rhinovirus genus [18]. These results are therefore in agreement with our earlier findings with the HRV-14 probe [2] and show that although there is considerable homology in the 5′ end non-coding region of many rhinoviruses it is still not sufficient for a probe prepared from this region to detect all the different rhinovirus serotypes with equal efficiency. Furthermore, in tests on clinical material, both the identity of the infecting serotype and its concentration in nasal secretion would vary widely. In this study there were great variations in the signal given by the different serotypes even though the titres of virus used were usually greater than 10^4 $TCID_{50}$/ml which is much higher than that normally found in nasal washing (often $< 10^2$ $TCID_{50}$/ml).

Recent work with synthetic oligonucleotides corresponding to short but highly conserved regions in the 5′ end non-coding region of the rhinovirus genome [18] suggests that such probes will detect all rhinovirus serotypes with equal efficiency [6]. Further studies are in progress.

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