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Detection of enteroviruses using cDNA and synthetic oligonucleotide probes

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Summary

This study compares the detection of enterovirus RNA by cDNA probes prepared from both the 5' and 3' end of the genome of coxsackie A21 and B4 with the use of synthetic oligonucleotides prepared from short but highly conserved sequences in the 5' end non-coding region of the picornavirus genome.

The cDNA probes detected enteroviruses with a variable level of sensitivity which presumably depended on the degree of genomic homology with the detecting probes. Generally probes from coxsackievirus A21 detected more enteroviruses than did similar probes from coxsackievirus B4. Probes from the 5' end of the genome of both viruses were more sensitive than 3' end probes. In contrast, synthetic oligonucleotides detected all enteroviruses efficiently suggesting that these probes could be useful as 'universal' probes to detect any enterovirus. This paper discusses the application of these probes in the diagnosis and differentiation of enteroviruses.

Picornavirus; Poliovirus; Echovirus; Coxsackievirus; Rhinovirus; cDNA; Probe; Synthetic oligonucleotide

Introduction

Picornaviruses are small RNA viruses, 23–27 nm in diameter. In those so far analysed the genome is a single-stranded RNA molecule of approximately
7200–7500 nucleotides which is polyadenylated at its 3′ terminus and covalently linked to a small viral protein, VPg, at its 5′ terminus (Ahlquist and Kaesberg, 1979). They comprise several species and numerous serotypes which infect man, animals and plants. Picornaviruses have been subdivided into four major genera: Enterovirus (poliovirus, coxsackievirus, echovirus), Cardiovirus (encephalomyocarditis [EMC] virus, mengo virus), Aphthovirus (foot-and-mouth disease virus) and Rhinovirus. Enteroviruses cause a number of serious illnesses in man including paralysis, meningitis and carditis while rhinoviruses are responsible for a substantial proportion of common colds in man (Melnick et al., 1979; Al-Nakib and Tyrrell, 1988).

The diagnosis of enteroviruses is usually based on virus isolation in a sensitive cell-line or in mice (Melnick et al., 1979). This is generally difficult, time consuming and requires expertise. Furthermore, once virus is isolated, supplementary tests are usually needed to identify and type the virus. Serological diagnosis of enterovirus infection is generally not satisfactory, although detection of virus-specific IgM can be useful in providing evidence of infection where the subgroup is known or suspected (McCartney et al., 1986).

Recently the genomes of a number of picornaviruses such as the polioviruses (Kitamura et al., 1981; Racaniello and Baltimore, 1981; Nomoto et al., 1982; Stanway et al., 1983; Toyoda et al., 1984; La Monica et al., 1986), coxsackie B3, B4 and A21 (Hughes et al., 1987; Jenkins et al., 1987; Lindberg et al., 1987) and rhinovirus 14, 2, 1B and 89 (Stanway et al., 1984; Skern et al., 1985; Duechler et al., 1987; Hughes et al., 1988) have been sequenced. A number of cDNA (Rotbart et al., 1984; Tracy, 1985; Al-Nakib et al., 1986) and RNA (Rotbart et al., 1988) probes have been generated and used in hybridization assays to detect these viruses. None has been found to be universally satisfactory. They show variable sensitivity for different picornaviruses, presumably due to variations in the degree of homology between the genome of the virus and that of the probe (Rotbart et al., 1984; Al-Nakib et al., 1986; Hyypia et al., 1987; Forsyth et al., in press). None has been found useful so far in detecting enteroviruses in clinical material.

Recently we have shown that because of the diversity of the rhinovirus genome, it is difficult to find an optimal cDNA probe to detect all Rhinovirus serotypes with equal level of sensitivity. However, synthetic oligonucleotides corresponding to short but highly conserved sequences in the 5′ end non-coding region of the genome of rhinovirus 14 detected all rhinovirus serotypes with equal efficiency (Bruce et al., 1988). Furthermore, they detected rhinoviruses directly in nasal washings from volunteers infected with a rhinovirus and the results, using these probes, correlated extremely well with those obtained by virus isolation (Bruce et al., in press).

In this study we report the application of these synthetic oligonucleotides to the detection of enteroviruses and compare the results with those using cDNA probes from both the 5′ and 3′ end of the genomes of coxsackievirus A21 and B4.
Materials and Methods

All echovirus and coxsackie A9 stocks were prepared by propagation in MRC-5 cells while those of coxsackie B viruses, polioviruses and coxsackie A7, A10, A16, A21 and A24 were prepared in Ohio HeLa cells. The titres of stocks were estimated by titration in microtitre plates as described previously for rhinoviruses (Forsyth et al., in press) and were greater than $10^4$ TCID$_{50}$/ml.

The coxsackie A viruses were a kind gift from Dr. Walker of the Public Health Laboratory at Epson. Echoviruses were obtained from the Public Health Laboratories at Colindale while the polioviruses were obtained from the Department of Microbiology at Odstock Hospital, Salisbury.

Details of the coxsackie cDNA probes were as follows. The coxsackie A21 5' end probe is a PstI-BamHI fragment (genomic positions 1-775) cloned into M13mp19. The 3' end probe is a EcoRV-PstI fragment comprising the final 790 nucleotides (positions 6211-7401). This was cloned into Smal-PstI M13mp18. On the other hand, coxsackie B4, 5' end probe consisted of a 531 base pair PstI fragment of the CB4 genome derived from cDNA fragment extending from the PstI site at the extreme 5' end of the genome to the PstI site at position 531, subcloned into M13mp18. The 3' end probe consisted of a 590 base pair PstI- BclI fragment of the CB4 genome subcloned into M13mp18. The Bcl site is at position 6805 in the CB4 genome and the Pst site is after the GC tail at the extreme 3' end of the cDNA clone.

The procedure for labelling these probes and conditions of hybridization employed in detecting enterovirus RNA using cDNA probes were the same as those recently described for rhinoviruses (Al-Nakib et al., 1986; Forsyth et al., in press). Hybridization signals were recorded as very strong (++++) , strong (+++), good (+), positive (+), weak (?) or none. These were estimated visually by two observers in the laboratory.

Synthetic oligonucleotide probe preparation

Two synthetic deoxyribonucleic acid probes were synthesized on a Biosearch 8650 automatic DNA synthesizer using B-cyanoethyl phosphoramidite chemistry. The first probe (GAGGCGGCGCGACTTACG), designated JWAER'1, is complementary to base numbers 452-468 of the nucleotide sequence of HRV-14 and the second probe (GATGAACCACCAAGCCA), designated JWAER'2, is complementary to base numbers 548-564 of HRV-14. 5' end labelling was carried out using T$_7$ polynucleotide kinase (PNK) and $\gamma$-[32P]deoxyadenosine triphosphate. Details of the methods used to label these probes have recently been described (Bruce et al., in press). Briefly, the reaction was carried out in a total volume of 100 µl containing 10 µl of 10 x PNK buffer (0.5 M Tris-HCl, pH 7.6, 0.1 M MgCl$_2$, 0.01 M DTT, 0.002 M EDTA), 4 µl (600 ng) of oligonucleotide, 10 µl $\gamma$-[32P]ATP and 10 µl PNK at 37°C for 1 h. Oligonucleotides were then separated from the $\gamma$-[32P]ATP on a 20 cm column of Sephadex G25. The specific activity of the probes was approximately 4-6 cpm/µg.
The procedure for hybridization has been described previously (Bruce et al., in press).

Results

Table 1 shows that cDNA probes prepared from the extreme 5' end of the genome of coxsackievirus A21 (CA21) and B4 (CB4) generally detected more enteroviral sequences.

| Virus   | Probe CoxA21 3' end | Probe CoxA21 5' end | Probe CoxB4 3' end | Probe CoxB4 5' end |
|---------|---------------------|---------------------|-------------------|-------------------|
| Cox A7  | 0                   | (±)                 | 0                 | (±)               |
| A9      | 0                   | 1                   | 0                 | (±)               |
| A10 (1) | 0                   | 1                   | 0                 | 1                 |
| A10 (2) | 0                   | 1                   | 0                 | 1                 |
| A16     | 0                   | (±)                 | 0                 | (±)               |
| A21     | 4                   | 4                   | 0                 | 1                 |
| A24     | 2                   | 4                   | 0                 | (±)               |
| Cox B1  | 0                   | 3                   | 2                 | 1                 |
| B2      | 0                   | 2                   | 2                 | 2                 |
| B3      | (+)                 | 3                   | (±)               | 3                 |
| B4      | 0                   | 1                   | 4                 | 3                 |
| B5      | 0                   | 2                   | 0                 | 1                 |
| B6      | 0                   | 3                   | 1                 | 2                 |
| Echo 1  | (±)                 | 2                   | 0                 | 2                 |
| 2       | 0                   | 1                   | (±)               | 1                 |
| 3       | 0                   | (±)                 | (±)               | (±)               |
| 4       | 0                   | 2                   | 0                 | 1                 |
| 6       | 0                   | 1                   | (±)               | (±)               |
| 7       | 0                   | 2                   | 1                 | (±)               |
| 8       | 0                   | 2                   | (±)               | (±)               |
| 9       | 0                   | 2                   | 0                 | (±)               |
| 11      | 0                   | 1                   | 0                 | (±)               |
| 16      | 0                   | 1                   | 0                 | 1                 |
| 19      | 0                   | 1                   | 0                 | (±)               |
| 20      | 0                   | 0                   | 0                 | 0                 |
| 22      | 0                   | 0                   | 0                 | 0                 |
| 25      | 0                   | 2                   | 0                 | 1                 |
| Polio 1 | 4                   | 4                   | 0                 | 1                 |
| 2       | 4                   | 4                   | 0                 | 1                 |
| 3       | 4                   | 4                   | 0                 | 1                 |

4 (+++++), Very strong hybridization signals; 3 (+++), Strong hybridization signals; 2 (+) Good hybridization signals; 1 (+), Positive hybridization signals; (±), Weak hybridization signals; 0, No hybridization signals.
viruses than those prepared from the extreme 3' end. This is in agreement with our previous findings with rhinovirus cDNA probes (Al-Nakib et al., 1986; Forsyth et al., in press).

Generally the 5' end CA21 probe detected more enteroviruses and with stronger hybridization signals than a similar probe from CB4. Both CA21 5' and 3' end probes gave very strong signals (++++) not only with the homologous virus but with poliovirus-1, -2 and -3 also. Furthermore, CA21 5' end probes gave very strong signals (++++) when reacted with RNA from CA24 virus, strong signals (+++) with RNA from CB1, -3, -6 viruses and good signals (+ +) with RNA from CB2, -5 and echovirus-1, -4, -7, -8, -9 and -25 viruses. In contrast to the findings with the 5' end probe, a CA21, 3' end probe generally reacted weakly with enteroviruses except for polioviruses and CA24 (signal ++).

Probes from the 5' end of the genome of CB4, reacted strongly (signal ++++) with RNA from CB-3 and -4 virus, and gave good signals (+ +) with RNA from

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**Fig. 1.** Enteroviruses RNA detection using synthetic oligonucleotide probes.
CB2, -6 and echovirus-1. On the other hand, a probe from the 3' end of the genome of CB4 gave a good signal (++) only when hybridized with RNA from CB-1 and -2 viruses. Generally, CB4 probes did not react well with the genomes of coxsackie A viruses, echoviruses or polioviruses.

In contrast to the findings obtained with cDNA probes, the synthetic oligonucleotides probes detected RNA from all the enteroviruses efficiently. Although, our initial experiments showed that the intensity of signals may vary, these clearly depended only on the amount of RNA contained in the samples (data not presented). It can be seen from Fig. 1 that the ratio of the intensity of the signal to the amount of RNA was constant for representative enteroviruses.

None of the control viruses such as coronavirus-229E, influenza A and B viruses, reovirus-1 and -3, parainfluenza virus-3 or herpes simplex virus gave a positive signal with these probes (data not presented).

These results were repeated several times and shown to be reproducible.

Discussion

The results of this study show that cDNA probes from both the 5' and 3' end of the genome of enteroviruses such as coxsackievirus A21 and B4 do not detect all enteroviruses with equal efficiency. The sensitivity of detection appears to depend on the strength of genomic homology between the probe and the virus under investigation.

These findings are in agreement with our recent findings on the genomic interaction between different rhinovirus serotypes using 7 rhinovirus cDNA probes (Forsyth et al., in press). They are also in general agreement with previous studies on the genomic interrelationship between enteroviruses using probes of different lengths and from different viruses (Rotbart et al., 1984; Tracy, 1985; Hyypia et al., 1988) and basically show that it would be difficult to select an optimal probe or a combination of cDNA probes that would detect all enteroviruses satisfactorily. This problem of sensitivity is further complicated by the fact that in clinical samples, these viruses are generally present in relatively low concentrations and therefore cDNA probes are unlikely to detect these viruses consistently or efficiently.

The problem of genomic heterogeneity affecting the sensitivity of picornavirus detection by cDNA probes was overcome in this study by using synthetic oligonucleotides corresponding to short but highly conserved sequences in the 5' end non-coding region of the picornavirus genome. Indeed, this and the similar study on rhinoviruses (Bruce et al., in press) show that these synthetic oligonucleotides are able to detect all picornaviruses efficiently irrespective of their genus or serotype. This suggests that such probes can be used as 'universal' probes to detect any picornavirus. It would, therefore, be interesting to generate other probes from sequences that may be typical of a certain serotype or a group of serotypes and in turn be used as 'typing' probes. This could be especially useful in differentiating for example, wild polioviruses from vaccine strains or polioviruses from other enteroviruses.
Our recent findings with rhinoviruses suggest that it is imperative to preserve samples immediately after collection in vanadyl ribonucleoside complexes (VRC) to prevent viral RNA degradation by endogenous RNAses (Bruce et al., in press). Indeed, collection and storage of samples in VRC improved detection rates considerably, and therefore, it is felt that collection and storage of enterovirus samples in these inhibitors of RNAses may also improve the detection rate of enteroviruses when using these probes.

We conclude that short synthetic oligonucleotides probes prepared from highly conserved region of the picornavirus genome may overcome the problem of genetic heterogeneity seen when cDNA probes are used to detect picornaviruses. We therefore, envisage that synthetic probes, as demonstrated for rhinoviruses, may prove useful in the detection of enteroviruses directly in clinical samples provided that these are collected and stored in VRC. We also anticipate that these probes will be extremely useful in the detection of viral RNA in situ in biopsy or autopsy material. Studies of these possibilities are now in progress.

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