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A cryptogram for recording rotavirus strains: the Rotacode

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SUMMARY

The RNA genome of rotaviruses consists of 11 segments in four size-classes which can be separated by polyacrylamide gel electrophoresis, although 11 separate bands are not shown by all strains. We propose a cryptogram (Rotacode) based on the relative distance of migration of adjacent bands in each size-class for coding the typical pattern of each strain of virus. This provides a shorthand for recording details of each strain and for grouping electrophoretically similar strains.

Rotacode was found to be reliable and reproducible, with identical codes being obtained for the same samples in repeated experiments under code and by various observers. Rotacode was also used to analyse 189 strains obtained over a three-year period and differentiated 13 electrophoretypes. This confirms the considerable electrophoretic variability of wild strains.

INTRODUCTION

The double-stranded RNA of rotaviruses has been found to be present in the virion as 11 segments which can be separated by polyacrylamide gel electrophoresis (PAGE) (Rodger, Schnagl & Holmes, 1975; Kalica *et al.* 1976; Todd & McNulty 1976; and numerous others). The patterns may vary between strains although individual strains give reproducible patterns (Kalica *et al.* 1976, 1978; Espejo *et al.* 1979). The cost and labour-intensiveness of electron microscopy and problems over the reliability of antibody-based tests to detect rotaviruses, exacerbated by the recent discovery of atypical strains which do not have the group antigen, has caused a rapid spread in the use of PAGE both for diagnosis and as a possible basis for classification.

Evaluation of PAGE results has been handicapped by the lack of a generally agreed system of nomenclature for the patterns of the RNA segments. Strict comparison between strains requires co-electrophoresis in one gel track of the strains concerned, an impossibly tedious task with every one of the huge number of recognizates being obtained throughout the world. A form of shorthand to group strains and allow some comparison between them is needed and previous methods of recording the patterns by drawing them (Lourenco *et al.* 1981; Pereira *et al.* 1983) have limitations in that 'patterns' cannot be easily listed or details exchanged between laboratories except by the use of the drawings or photographs.

In this paper we propose the Rotacode to fill this gap. This is a system of coding the patterns using numbers and letters so that the information can be transmitted

using only a typewriter. We have used the code on a considerable variety of strains and found it both useful and reproducible. It is purely descriptive and use of it does not imply that strains with the same code entries are necessarily the same. Considerable additional work will be needed to make the necessary detailed comparisons. Already it is evident that strains with apparently indistinguishable patterns (electro-phoretypes) in one-directional gels can show differences when fully analysed after digestion with ribonucleases (Clarke & McCrae, 1982) but more and more routine laboratories are using one-directional PAGE for routine diagnosis of rotavirus infections without prior enzymic digestion (see, for example, the Communicable Diseases (Scotland) Report during 1984). The Rotacode recognizes an existing need and, by grouping together strains with broadly similar RNA patterns, will indicate where further comparisons should be made. Such comparisons can then establish any significant differences.

Rotacode, because it is purely descriptive and does not number the segments, makes no judgements on the patterns observed. It can be used, for example, on strains whose RNA patterns do not show all 11 segments without any need to decide whether the missing segments are absent or just coincident with other segments. As further atypical strains are found, the code may have to be adapted but it should prove to be sufficiently flexible. We have used it satisfactorily to analyse the rotavirus strains obtained in Newcastle between 1980 and 1983.

MATERIALS AND METHODS

Nomenclature

It is well accepted that the rotavirus genome consists of 11 segments of double-stranded RNA. However, it is comparatively rare to obtain 11 separate segments on PAGE. Hence an individual band may represent more than one segment and we have used the term band to refer to a visible line on the gel. It would be incorrect to use segment and we feel that line could be misleading by implying a single component.

Specimens examined

Stool specimens were obtained from infants and children, from hospitals and from a nursery in the north-east of England over the period October 1980 to June 1983. A 10 % extract of the stool was made in Hanks's Balanced salt solution (BSS), well shaken and clarified at 1500 rev./min for 10 min in an MSE Mistral 4L centrifuge. The supernatant was used for RNA extraction.

Cell culture virus

Cell culture-adapted rotavirus strains of human (Wa) and bovine origin were kindly supplied by Dr B. Totterdell (St Thomas's Hospital Medical School, London). The viruses were grown in the continuous rhesus monkey kidney cell lines MA 104 and LLC-MK2 respectively, in the presence of crystalline trypsin (Armour, 20 μg/ml). Infected cultures grown in Medium 199 without serum were harvested after 3 days, frozen to −20 °C and thawed to 37 °C three times and clarified at 3000 rev./min for 10 min at 4 °C in an MSE Super Minor Centrifuge. The supernatant was then used for RNA extraction.

Deproteinization of viral nucleic acid

Initially viral RNA was extracted from the clarified suspensions (stool and cell culture) by treatment with 10 % sodium dodecyl sulphate (SDS), freshly re-distilled phenol and ethanol-salt precipitation as described by Rodger, Schnagel & Holmes (1975). The RNA precipitate was resuspended in 50 ml sample buffer (62 mM tris-HCl, pH 6·8 containing 3 % SDS, 5 % 2-mercaptoethanol and 40 % glycerol) for electrophoresis.

In later experiments unclarified stool extracts or infected cell-culture harvests were used. Aliquots of unpurified virus were treated with 10 % SDS and incubated at 37 °C for 30 min to release viral nucleic acid. The treated extract was then mixed with an equal volume of sample buffer for electrophoresis.

Polyacrylamide gel electrophoresis of RNA

Electrophoresis of deproteinized RNA was conducted in 1·5 mm thick 7·5% polyacrylamide slab gels with a 3 % stacking gel, using the discontinuous buffer system described by Laemmli (1970).

Electrophoresis was done at the temperature of cold tap water (about 17 °C) overnight (approximately 16 h) at a constant current of 30 mA.

Gels were stained with silver nitrate using minor modifications to the method published by Herring *et al.* (1982). The staining time in silver nitrate was reduced by 0·5–1 h and the gels were stained in suspension after removal from the supporting glass plates. The solutions were used without prior degassing and the stained gels were photographed by transmitted light over an X-ray viewing box using 35 mm Ilford Pan F film and a 55 mm Micro-Nikkor lens.

RESULTS

Development of the Rotacode

Fig. 1 shows the RNA pattern of a rotavirus showing all 11 segments as discrete bands. As indicated, these may be divided into four size classes: class I (segments 1-4), class II (segments 5 and 6), Class III (segments 7-9) and class IV (segments 10 and 11). Within each class the individual segments from different strains of rota virus may show variations in the distance migrated relative to each other. Overlapping is common so that several segments may form a single band but the whole pattern is characteristic for each strain and the Rotacode, by providing a notation for each size class, provides a shorthand version.

Each class is considered in turn, as indicated below, and the four notations are combined to form the Rotacode for that strain. Reference to Fig. 2 in reading what follows will help the reader to understand the entries.

Class I. Major variations in bands 1 and 4 are uncommon, but they may be found in bands 2 and 3. These latter bands may occur as:

		Rotacode notation
(i)	A single band	1
(ii)	Two separate bands which may be	
	(*a*) Close together or	2C
	(*b*) Further apart	2F

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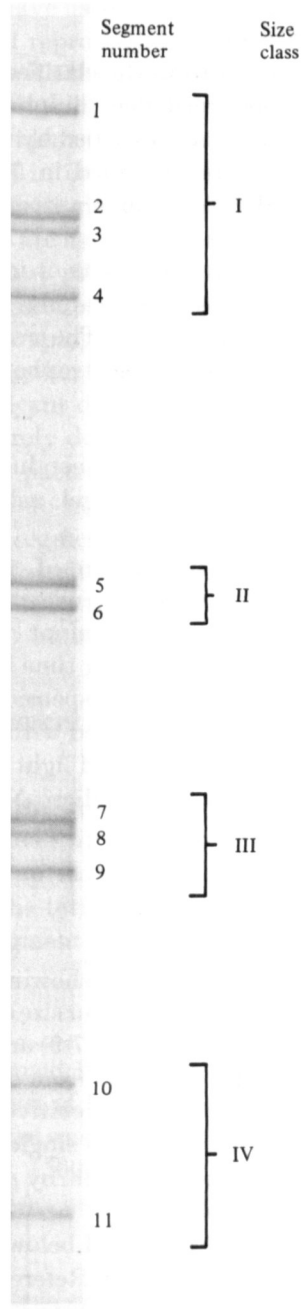

Fig. 1. Rotavirus RNA profile showing all eleven segments (1–11) in four size classes (I–IV) as indicated.

The variations in bands 1 and 4 do not yet appear to be consistent enough to warrant a specific notation.

***Class II*. These are usually present as two bands separated by a space which may be**

	Rotacode notation
(i) narrow, or	**N**
(ii) wider apart	**W**

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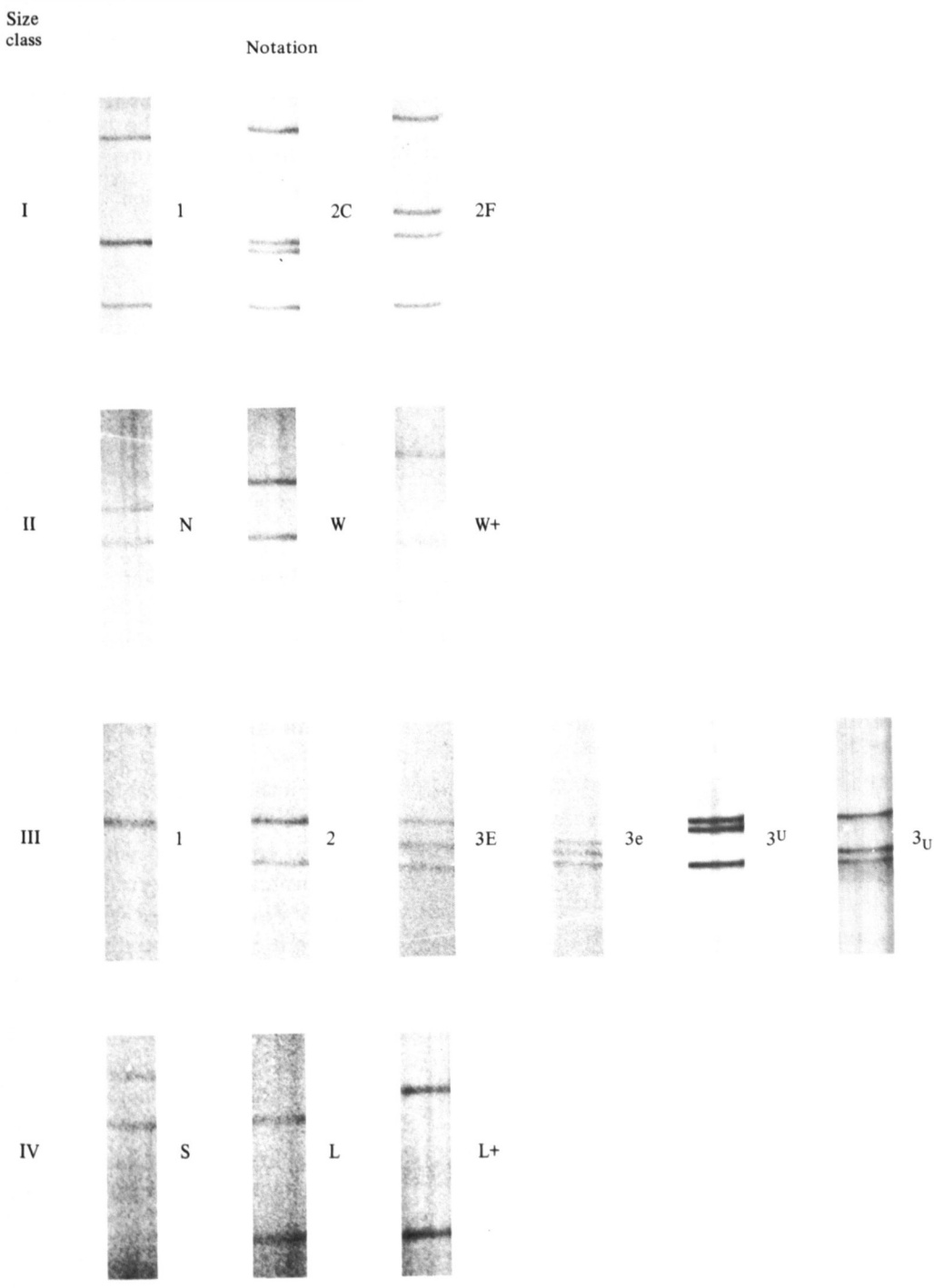

Fig. 2. Variations in the four size classes with their corresponding Rotacode notations.

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Occasional strains show a wider than usual space, requiring an additional notation W^+ (Fig. 2). So far W^+ strains have been found only in association with a short pattern is size class IV.

Class III. This class shows the most variation with the segments appearing as one, two or three bands. When three bands are seen, they may be equally or unequally spaced. These alternatives are given the following notations.

		Rotacode notation
(i)	One band	1
(ii)	Two bands	2
(iii)	Three bands, equally spaced which may give a broad pattern, or a	3E
	narrow pattern	3e
(iv)	Three bands, unequally spaced	
	(*a*) Band 8 nearer band 7	3^U
	(*b*) Band 8 nearer band 9	3_U

Class IV. The variation here has been well described as giving short or long patterns (Espejo *et al.* 1979). In the long pattern band 11 is well separated from 10 and the Class III bands. In the short pattern the gap is substantially less and the two alternatives are not readily confused.

		Rotacode notation
(i)	Long pattern	L
(ii)	Short pattern	S

Occasional strains are found which have a basically long pattern but whose band 10 migrates more slowly than usual (Fig. 2), giving a greater space between bands 10 and 11. The Class IV notation may therefore include an additional notation, L+.

The complete Rotacode notation for each strain of virus has four components, one for each size class. This is illustrated in Fig. 3, which shows the RNA pattern of the standard strain Wa. Inspection of the figure, and comparison with Fig. 2, shows that it has a Rotacode of 2C, W, 3E, L. The commas are inserted here for clarity but we feel that they can, and usually will, be omitted once the user is familiar with the system. Initially it will be necessary to include a standard strain (such as Wa) in each gel but we have found that, with a little practice, it is possible to code new strains by direct inspection.

Rotacode can be used to show one or many variations in RNA profiles. Stool extracts VJ 4740 and VJ 3503 (Fig. 4*a*) have Rotacodes 1, W, 2, L and 2C, W, 2, L respectively. The difference in the two patterns occurs in size class I, where bands 2 and 3 occur as a single band in one strain and as two separate bands in the other. This single difference is reflected quite clearly in their Rotacodes.

Samples VJ 1391 and VJ 3222 (Fig. 4b) have the Rotacodes respectively of 2F,

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Fig. 3. RNA profile of the Wa strain of human rotavirus which has the rotacode 2C, W, 3E, L.

Fig. 4. Rotacode in practice. (*a*) Two strains of rotavirus, VJ 4740 (left track) and VJ 3503 (right track), with rotacodes 1, W, 2, L and 2C, W, 2, L respectively, showing a single difference (in Size Class I). (*b*) Two strains, VJ 3222 (left track) and VJ 1391 (right track), with rotacodes 2F, W, 1, L and 1, W^+, 2, S respectively, showing differences in all four size classes.

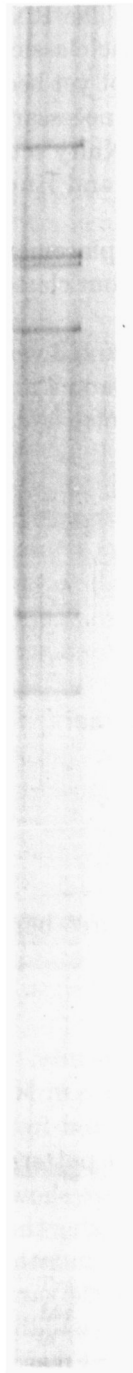

Fig. 3.

Fig. 4.*a*.

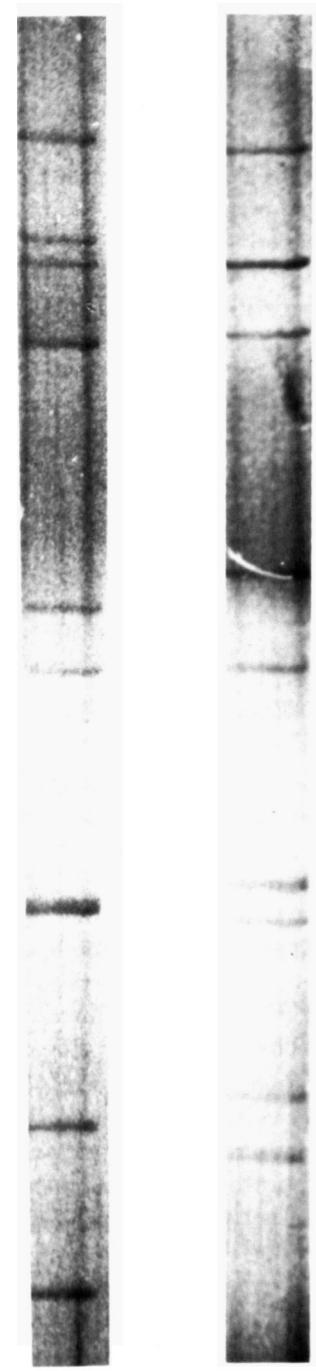

Fig. 4.*b*.

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W, 1, L and 1, W+, 2, S and show variations in each of the four size classes. Again these differences are apparent by comparing their Rotacodes.

Atypical rotaviruses

Recently strains lacking the rotavirus group antigen have been recovered, firstly from birds and animals but subsequently from human faeces as well. The RNA patterns shown in PAGE show substantial differences from those of the classical rotaviruses described so far in this paper. These atypical strains have not yet been shown to be common but some additions to the Rotacode notations are necessary. Those which follow are based on published papers by, for example, McNulty *et al.* (1981), Bohl *et al.* (1982), Dimitrov *et al.* (1983), Snodgrass *et al.* (1984) and Hung Tao *et al.* (1984).

The atypical strains which have typical rotavirus morphology show displacement of several RNA segments into other size classes although, broadly, the four classes can still be discerned:

Class I. Bands 2 and 3 may appear more separated than usual with band 3 very close, in some cases, to band 4. This requires an extra notation 2F+. Band 4 also shows greater variability but it is not yet clear what extra notation(s) is/are necessary.

Class II. This may consist of 1 or 3 bands:

		Rotacode notation
(i)	One band	1
(ii)	Three bands, equally spaced	3E
(iii)	Three bands, unequally spaced, usually with band 6 closer to band 7 than band 5	3_U

Class III. These do not usually appear as 3 bands. They may appear as:

		Rotacode notation
(i)	One band	1
(ii)	Two bands	
	(*a*) Narrow pattern	2N
	(*b*) Wide pattern	2W

Class IV. This may be seen as the usual long pattern (L) or as a three band pattern based on the long type 3L.

Rotacode in practice

The virtue and value of Rotacode was evaluated in a series of experiments.

Reproducibility in PAGE. The Wa strain has been passed several times in MA 104 cells over the past 2 years in this laboratory. The RNA was extracted from representative harvests as indicated under Materials and Methods and the patterns separated simultaneously on parallel tracks in a single gel. The results are shown in Fig. 5. The Rotacode for all passage levels was 2CW3EL. This indicates that Wa gave a consistent Rotacode over 21 passages in cell culture over 23 months. We have also found that wild strains with one exception (see below) gave the same pattern when re-extracted. With material limited by the amount of stool available we have not tested this stability exhaustively but we have no substantial reason doubt it, as shown below, and others have also found considerable stability.

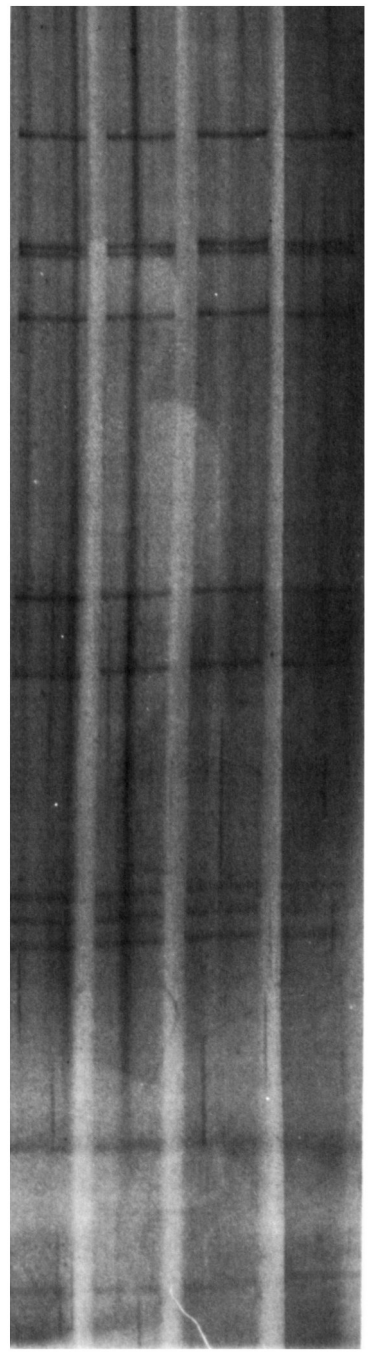

Fig. 5. RNA profiles of strain Wa at different passage levels. Track 1 (reading from left to right); Harvest in Feb. 1982 (after six passages of virus from Dr Totterdell). Track 2: harvest in June 1982 (passage 9). Track 3: harvest in Jan. 1983 (passage 14). Track 4: harvest in Jan. 1984 (passage 21). All passage levels have the same 2CW3EL rotacode, and no variation was detected.

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Table 1. *Analysis of 36 coded specimens by Rotacode*

	Rotacode	Number of specimens designated		
		Originally	By observer 1	By observer 2
1	1, W, 2, L	3	3	3
2	2F, W, 1, L	10	10	10
3	2F, W, 2, L	1	1	1
4	2C, W, 2, L	5	5	5
5	2C, W, 3E, L	2	—	—
6	2C, N, 3^U, L	2	1	1
7	2C, N, 2, L	0	1	1
8	2C, W, 3^U, L	1	1	1
9	2C, W, 1, L	2	1	1
10	Negatives	10	13	13
	Total	36	36	36

Reproducibility in reading. This experiment was designed to show that our PAGE method was constant in sensitivity and that we could apply the code consistently. Thirty-six specimens (26 positive for rotavirus and 10 negative) were coded by one of us (C.R.M.) and given to another (R.B.M.) for extraction and electrophoresis on two gels at the same time. The results were read and coded independently by two observers (R.B.M. and R.A.) before the code was broken. The positive materials included 22 stool extracts, cell culture grown Wa (twice) and a bovine strain (twice) also grown in cell culture. The results are shown in Table 1, from which it can be seen that the patterns given by the stool extracts, were all given the same code by both observers neither of whom knew what the previous code had been or the other's assessments. With one exception these also agreed with the original coding. The exception, read by both operators as 2CN2L, was subsequently tested a fourth time and again gave the result of 2CN2L. It seems that the original coding of $2CN3^UL$ may have been a mistake or a solitary occasion when bands 7 and 8 were separated. Of the cell culture extracts, three out of the four failed to give readable patterns while the fourth again gave the typical 2CW1L code of our bovine strain. These cell culture materials were evidently less stable on storage, a problem we have also encountered with extracts of stools stored at −20 °C but not with unextracted faeces stored at the same temperature.

Coding of wild strains, 1980–1983. During this period, 189 strains were identified in faecal specimens sent to this laboratory. They included specimens from babies in hospital in Newcastle, in the University day nursery and from a longitudinal survey of babies at home in Gateshead. Table 2 indicates the variety of Rotacodes found and the numbers of each found in each year. Thirteen different Rotacodes were recorded but with three (2CW2L, 2FW1L and $2CW3^UL$) accounting for 166 (88%). The remainder (12%) were found relatively occasionally with no other code accounting for more than eight (4%). 2CW2L was the predominant strain (92 = 49%) and was found repeatedly in all three years, for which it accounted for 75%, 24% and 55% of the strains identified in the respective years. The only year in which it did not form more than half of the recognizates was 1981–2, in which 2FW1L strains predominated. $2CW3^UL$ only appeared in substantial numbers in 1982–3, in which 27% had this code. No atypical strains were found.

Table 2. *Use of Rotacode to identify electrophoretypes* (1980–1983)

Electrophoretype Number	Rotacode	Number identified in 1980–1	1981–2	1982–3	Total
1	2C, W, 2, L	30	16	46	92
2	2F, W, 2, L	4	—	—	4
3	2F, W, 1, L	—	48	—	48
4	2C, W, 1, L	—	—	2	2
5	2C, N, 1, L	—	—	1	1
6	2C, N, 2, L	—	—	2	2
7	2C, N, 3^U, L	2	—	6	8
8	2C, W, 3^U, L	4	—	22	26
9	2F, N, 3^U, L	—	—	1	1
10	1, W^+, 2, S	—	—	1	1
11	2C, W^+, 3E, S	—	—	2	2
12	1, W^+, 3^U, S	—	1	—	1
13	1, N, 3^U, L	—	1	—	1
	Total	40	66	83	189

The numbers coded so far are still small and we have not tried to draw any conclusions from these results. They show that a considerable number of different codes may be identified in one area over a period of 3 years, and that the code may be used to bring out the patterns of infection. Interestingly, some codes were only found in RNA extracted from stored stools obtained in Glasgow in the 1970s (data not shown), and raises the possibility that geographical variations may also be shown through the use of Rotacode.

DISCUSSION

Rotaviruses are among the commonest viruses in the world, readily detectable by several techniques. The discovery of strains which do not possess the group antigen in several species, including man, has meant that techniques which do not depend on antisera to detect virus will be valuable. Polyacrylamide gel electrophoresis has shown that the RNA segment patterns vary between strains and can form part, at least, of any classification system, particularly because the PAGE technique has now been simplified and made more sensitive by using the silver-staining technique.

Although differences have been noted by several workers, comparisons have been hampered by the lack of an agreed nomenclature with which to make them. It is still uncertain how different one pattern must be from another for the difference to be significant. The Rotacode we propose in this paper is not intended to take note of very small differences but is intended more to provide a convenient shorthand to group together strains with broad similarities. More sensitive techniques, such as oligonucleotide mapping, can be used on strains with the same Rotacode to look for further differences.

At present workers in a rapidly expanding number of laboratories are using PAGE both to identify rotaviruses in stool extracts and to make comparisons

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between the strains they identify. In the absence of any cryptogram to codify their findings each is, in effect, working in isolation. Rotacode could provide a common language and, because it allows the reader to reconstruct the RNA pattern found without actually seeing it, should prove useful in establishing broad epidemiological patterns.

Previous attempts to put rotaviruses into groups by RNA profiles have used single-letter non-descriptive codes (Rodger & Holmes 1979; Lourenco *et al.* 1981). Rodger & Holmes (1979) proposed a system similar to that used for strains of influenza virus (a letter for the group and a serial number for the strain). This system is useful to the reader only if he is familiar with each group pattern and he can deduce nothing from the nomenclature itself. It also has the disadvantage that new patterns can only be added by agreement (i.e. someone will have to act as a clearing house or recorder), and workers discovering possible new patterns will not be able to code them until their existence has been acknowledged and a new letter allocated. Rotacode does not suffer from this disadvantage since new strains can be coded instantly with no need for them to take their place in a queue.

Lourenco *et al.* (1983) made drawings of their patterns and assigned a single letter to new patterns and, in their system, strains with variations in Class III were not given different code letters.

But does Rotacode work? Our preliminary results with it suggest that it does. With one exception, we have found it reliable and reproducible and, where re-coding was different from the original, the difference was small and may have been due to an original miscoding. Otherwise the same gels read by two separate observers were given the same code. Except for the one strain mentioned their results were identical but problems were experienced with the cell culture-grown standard human and bovine strains. These were not due to variable codes but failure of the extracts to give line patterns at all after storage. We think it is important to include standard strains in gels for coding (at least at first) and it will be necessary to solve the problem of storage of suitable extracts.

Our initial use of Rotacode for epidemiology has revealed some interesting patterns. Strains with 13 different codes were recorded during 3 years in the Newcastle area and further data (not included here) from stored specimens from Glasgow between 1974 and 1979 suggest that other electrophoretypes were current there. With strains being recovered from both normal babies and those with diarrhoea it seems probable that strains varying in pathogenicity will be identified in due course. If these differences are reflected in electrophoretypes they could be shown by the use of Rotacode, for which it could have just the right degree of sensitivity.

In its present version, Rotacode takes note of only nine out of the eleven segments. We have not found enough consistent variation so far in bands 1 and 4 to warrant code entries. Other workers may find a need to modify the code to record these variations. If so, the first entry in the code will have to be altered. We expect Rotacode to be modified in use as new variations in the PAGE pattern are found. We do *not* think it should take note of all fine differences. Its strength is, we believe, that the cryptogram for any strain can be deduced easily by inspection and no elaborate measurement is needed.

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