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DETECTION OF ROTAVIRUS INFECTION BY IMMUNODIFFUSION

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ABSTRACT

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Three precipitin reactions associated with bovine rotavirus infection were demonstrable by immunodiffusion. One of the reactions has been utilized in a diagnostic test for the detection of rotavirus in faeces, or specific antibody to rotavirus group antigen in serum or faeces. The test, based on bovine materials, appeared to be group-specific and effective in demonstrating rotaviral antigen or antibody in other species of animals, including human beings. The procedure was as efficient as electron microscopy in detecting evidence of rotavirus in faeces of calves and a range of other species.

INTRODUCTION

Electron microscopy (EM) is a standard procedure for the detection of rotavirus (RV), but its routine use for diagnosis is limited by costs, time and equipment. Alternative methods which have been employed include immunoelectron-microscopy (IEM), immunoelectro-osmophoresis (IEOP), complement fixation (CF), radioimmunoassay (RIA), enzyme-linked immunoabsorbent assay (ELISA) and immunofluorescence (IF) (Flewett and Woode, 1978). Woode et al. (1976) demonstrated cross reactions among RV of different species by immunodiffusion (ID) using a group antigen associated with the inner of the two capsid layers. However, they found ID to be of low relative sensitivity compared to EM. Mathan et al. (1977) successfully used ID to test faeces or sera for RV antigen or antibody respectively, but did not indicate the sensitivity of the test. Mohammed et al. (1978) compared the ability of counter-immunoelectrophoresis (CIEP), CF, haemagglutination and ID to detect various RV antigens. They found all tests, except CF, to be more sensitive in detecting soluble antigen than other antigens, and CIEP to be the procedure of choice for routine testing. Rhodes et al. (1979) compared ID, IF and EM for antigen detection and found more positives were detected by ID and EM.

The present paper reports the details of an ID procedure for the detection of RV and antibody, and compares the results of ID with those obtained by EM.
MATERIALS AND METHODS

Immunodiffusion procedure

Agarose gels were cast in disposable 90 mm Petri dishes, 12 ml per plate. Six hexagonal patterns were cut in each plate. Each pattern consisted of six outer wells placed 8 mm, centre to centre, from a centre well. All wells had a diameter of 6 mm. Reference antigen was placed in the centre well and reference antiserum in three alternate outer wells. Test samples, either faecal material or serum, were placed in the intervening wells. An automatic pipette with disposable 0.05 ml tips as used to fill the wells. The plates were covered, placed in a humid chamber at room temperature, and read 24 h later.

Thus three reference lines (Fig. 1, line D) of one specificity were provided within each pattern and there was opportunity for each test sample to modify or deviate one end of each of two reference lines.

The balancing of reference antigen and antiserum (as below) was performed in agarose concentrations of 0.5%, 0.75% and 1%, each in barbital buffer pH 7.4 with 0.5% CdCl₂ and 0.125% glycine (Crowle, 1973), in 0.02 M phosphate buffered saline pH 7.2, or in borate buffer pH 8.6 consisting of 2 g NaOH, 9 g H₃BO₃, 0.1 g NaN₃, 10 g agarose and distilled water to 1 l.

Preparation of reference antigen and antiserum

Initial reference antigen and antiserum were obtained from two calves which were experimentally infected when one day old, by oral dosing with faeces containing RV from a field case of calf scours. Diarrhoea commenced 2 and 3 days later and continued for one week. Thirteen faecal samples were collected from each calf, one or more daily during the period of diarrhoea. RV was detected in the faeces by EM for the first 5 days of diarrhoea. Two weeks after diarrhoea ceased one of the calves was inoculated subcutaneously with a preparation of virions from a 1.36—1.38 g/ml CsCl fraction of its own diarrhoeic faces. This calf was bled 26 days after injection for production of reference antiserum.

The initial reference antigen was derived from pooled EM-positive faeces from the experimentally infected calves. The faeces were diluted to a liquid consistency with distilled water. A portion of this mixture was homogenized in an equal volume of trichlorotrifluoroethane¹ (TCTFE) and centrifuged at 10,000 g for 30 min. Supernatant was layered onto a 30 ml preformed 1.25—1.38 g/ml CsCl density gradient and centrifuged overnight in a Beckman SW27 rotor at 82,000 g (av) at 5°C. The 1.36—1.38 g/ml fraction was stored without preservative at 4°C for 4 weeks before its initial testing against the antiserum.

The optimum dilutions of reference antigen and antiserum were found by diffusing two-fold dilutions of antigen each against a similar series of serum

¹ Arklone-P, Imperial Chemicals Industry Limited
The highest dilutions of antigen and antiserum giving a distinct centrally placed precipitin line of adequate extent were used in subsequent tests to form the reference line against which faecal or serum samples were tested. The reference antiserum dilution thus determined was subsequently used as the standard against which other batches of reference antigen were balanced.

Further batches of reference antigen were prepared from ID-positive faecal samples by a simpler procedure, in which faeces were homogenized in an equal volume of TCTFE and centrifuged at 10,000 g for 30 min. The clear supernatant was usually suitable; it was tested by ID and the concentration adjusted, so that, when diffused against the standard dilution of reference serum, it gave a reference line equivalent to that of the initial reference antigen. This sometimes required adjustment of its activity by appropriate dilution, or concentration by dialysis against polyethylene glycol (PEG) MW 20,000. In some cases it was necessary to further purify the supernatant by CsCl density gradient centrifugation to remove excessive non-specific precipitation.

**Examination of faecal samples by ID and EM**

Faecal samples were diluted, when necessary, to a liquid consistency with distilled water, treated with TCTFE and centrifuged at 10,000 g for 30 min. The clear supernatant was tested by ID. For EM, 4 ml of the supernatant was centrifuged at 100,000 g for 2 h in a Beckman SW60 rotor at 5°C. The resulting pellet was resuspended in 0.1 ml distilled water, mounted on a formvar—carbon-coated grid, negatively stained with 1% ammonium molybdate, pH 5.3, and examined in a Philips 201 or 300 transmission microscope. Occasionally, preparations had to be further purified and were centrifuged through 30% sucrose. The EM preparation was also tested by ID.

Faecal samples examined (Table I) included those from field cases of diarrhoea in calves (153), pigs (57) and human beings (2) and also those (supplied by Dr. S. Tzipori) from experimental infections in calves (28), foal, pig and mouse (one each).

Serum samples from 100 adult cattle, nine dingoes and nine feral pigs, all selected at random from material submitted to the laboratory, together with small numbers of sera from various host species (supplied by Dr. S. Tzipori) after experimental infection (Table II), were tested by ID.

**Separation of RV precipitating antigens**

Faecal samples with high RV content by EM were prepared and layered onto a CsCl gradient and centrifuged as previously described. The gradients were collected and the density of each fraction calculated from refractive indices. Locations of virions were determined by EM. Fractions were dialysed against distilled water overnight at 4°C then tested by ID against undiluted antiserum to locate rotavirus antigens.
TABLE I

Comparison of the results from EM and ID examination for virus of samples from calves and pigs with diarrhoea, and from a range of experimentally infected animals

| Faecal sample | EM-positive |   | EM-negative |   |
|---------------|-------------|---|-------------|---|
|               | ID-positive | ID-negative | ID-positive | ID-negative |
| Field cases   |             |             |             |             |
| Calf          | 37          | 0           | 0           | 116¹       |
| Pig           | 11          | 1           | 0           | 45         |
| Human         | 2           | 0           | 0           | 0          |
| Experimental infections |             |             |             |             |
| Calf          | 18          | 0           | 0           | 10         |
| Foal          | 1           | 0           | 0           | 0          |
| Pig           | 1           | 0           | 0           | 0          |
| Mouse         | 1           | 0           | 0           | 0          |
| Total         | 71          | 1           | 0           | 171        |

¹ Coronavirus was seen in two of these samples by EM

TABLE II

Results from the application of ID for the detection of antibody in serum collected at random and from animals recovered from confirmed infection with RV

| Serum donor | Positive-ID | Negative-ID |
|-------------|-------------|-------------|
| Random      |             |             |
| Adult cattle| 100         | 0           |
| Dingo       | 5           | 4           |
| Feral pig   | 8           | 1           |
| Recovered   |             |             |
| Calf        | 2           | 0           |
| Foal        | 1           | 0           |
| Pig         | 1           | 0           |
| Mouse       | 1           | 0           |

RESULTS

Diagnostic immunodiffusion

Adequate reference lines were produced when the antiserum used throughout this work was diluted to 1/2 and reacted with reference antigen. The reference line was clearest in 1% agarose in barbital buffer.

Additional precipitin lines were occasionally obtained from some antigen batches and faecal preparations tested. In reading the routine test, these
were regarded as non-specific as they failed to form turns of identity with the reference line. However, subsequent evaluation of these by determining their presence in normal or experimentally infected animals, and their association with EM demonstrable virus, indicated that most were not related to RV although two were probably due to precipitation of the RV virion antigens described below.

The results of the application of the test for the detection of rotaviral antigen in faeces are given in Table I. This shows that ID detected the presence of RV in all but one of the cases in which RV was detected by EM. When used to detect antibody (Table II), all serum samples from adult bovine animals were positive with 51 out of 100 giving reactions as strong, or stronger, than the reference serum. The majority of dingo and feral pig sera were also positive. Sera from a range of experimentally infected animals were all positive in the test.

Antibody was demonstrated in the faeces of 15 of the 116 field cases of bovine diarrhoea in which antigen was not detected.

When samples concentrated by centrifugation for EM were examined by ID, results did not differ in the routine test from those obtained on the same samples which had not been centrifuged. However, other RV antigens were more commonly recognized in the centrifuged samples.

A small number of samples was tested as raw faeces. Broad zones of non-specific precipitation made the test unreadable.

Other precipitating antigens of RV

After centrifugation the CsCl gradient covered 1.17—1.41 g/ml. Two light scattering bands were visible at levels subsequently determined to be 1.36 and 1.38 g/ml.

Examination of all fractions by EM revealed some disrupted virions and fragments throughout the gradient. Whole double capsid virions were present in large numbers in the 1.36 g/ml fraction and single capsid virions in the 1.38 g/ml fraction.

By ID three precipitating antigens were demonstrated (Fig. 1). Fractions from the range 1.23—1.28 g/ml gave a single line identical to the reference line of the diagnostic test, and this antigen appears to be "soluble". Lesser levels of the same activity appeared throughout the gradient. Fractions containing the 1.36 and 1.38 g/ml bands gave single lines closer to the wells, which were quite separate from the reference line of the diagnostic test.

When the separate lines were cut from the gel, homogenized lightly in distilled water and viewed by EM, no virions were associated with the line derived from 1.23—1.28 g/ml fractions, while double capsid virions were numerous in the line derived from the 1.36 g/ml fraction. The line from the 1.38 g/ml fractions was faint and no morphological entity associated with this line was recognized.
The three antigens were subsequently identified in faecal extracts, the "soluble" antigen was active in relatively dilute, and the virion antigens only in more concentrated, preparations.

DISCUSSION

Three precipitin reactions were demonstrated when CsCl-fractionated RV-positive faeces were diffused against RV-positive antiserum. Of the three precipitating antigens, two could be directly correlated with RV virion, one with a double capsid from a 1.36 g/ml density fraction and the other with a single capsid from a 1.38 g/ml density fraction. Due to the poor diffusion rate of particulate antigens, each of these reactions occurred close to the antigen well and thus was not suitable as reference antigen for diagnostic work. The third antigen, from a 1.23—1.28 g/ml fraction, did not contain virions demonstrable by EM. It diffused more rapidly than the other two antigens and precipitation occurred near the middle of the reaction zone. It had the practical advantage of being demonstrable in higher dilutions of viral or infected faecal extracts than were the two virion antigens. It provided a suitable reference line against which to test samples of unknown reactivity, and was selected as the reference antigen.

"Soluble" antigen was originally identified in a 1.36—1.38 g/ml fraction.
containing virions, which had been held at 4°C for 4 weeks without preservative. In other CsCl gradients it was found predominantly in a 1.23–1.28 g/ml fraction, but was also present in small amounts in all fractions. The relationship between “soluble” and virion is not clear.

Specificity of the test was shown to be broad, encompassing RV infections of a wide variety of hosts and a number of RV virus types or subtypes distinguishable by virus neutralization (Dr S. Tzipori, personal communication). As it seems that RV types may vary within a single host species (Thouless et al., 1978; Zissis and Lambert, 1978), the broad specificity of the test is an advantage in many circumstances where discrimination within the RV group is not required. The epidemiological specificity (Martin, 1977) of immunodiffusion is high. In the present work it is not demonstrably less than 1.00.

The sensitivity of the test also appears to be high. No false negative results were obtained from 55 bovine samples which contained rotavirus demonstrable by EM. When samples from all species were included, ID positives were obtained on 71 of 72 EM-positive samples, giving a nominal relative sensitivity compared to EM of > 0.98.

Many studies have been reported in which various techniques for the demonstration of RV have been compared (Flewett and Woode, 1978). When ID has been included, the procedure used has generally been of low sensitivity. Woode et al (1976) found only 55/148 EM-positive samples were detected by ID, a relative sensitivity of 0.37. Some points of procedure should be noted as important for the better results obtained from ID in the present work. It is probably most important that the sample be tested in the presence of known positive antigen and antibody, between which a reference line of precipitation is formed. The dilutions of reference reactants should be carefully chosen to produce a line of maximum sharpness and extent just sufficient to reach the well containing the test sample. The use of a reference line in ID is essential to establish the specificity of a reaction, and according to its strength determines the sensitivity of the test.

Sensitivity is improved because reactivity is detected by deviation of the reference line. This deviation may be produced by about one-tenth of the activity required for a convincing independent precipitation by the test sample against the appropriate reactant (Littlejohns, unpublished). About 30% of ID-positive faecal extracts in the survey turned the reference line but did not extend the precipitation between extract and positive serum wells, i.e. no reaction would have been recorded in a simple, two-well test between extract and serum. The proportion would undoubtedly be less if a stronger serum were used.

The immunological specificity of the precipitin reaction is guaranteed by the use of a reference line. This is important because experience indicated that non-specific precipitation between faecal extracts and the serum was common, and must be distinguished from the chosen specific reaction.

Although barbital buffer was used throughout this work, more recent experience suggests that borate-buffered gel might be preferable for use with
antigens prepared without density gradient purification. Non-specific reactions from these crude antigens seem less apparent in borate-buffered gel.

Detection of antigen and antibody, in faeces and sera respectively, from RV infections in other host species indicates that the test is RV group-specific. This is consistent with ID reactions previously reported, in which the inner capsid is described as the source of group-specific antigen (Woode et al., 1976). The description of whole (double capsid) virion agglutination by type and specific neutralizing sera, and the deduction that type-specific antigenic activity is located in the outer capsid (Bridger, 1978), suggest that the precipitin reaction involving double capsid virions may also be type specific. The use of this reaction for diagnostic purposes is limited, at least in the patterns used routinely, by the poor diffusion of the antigen. It could possibly be used in patterns designed to straighten the reference line, or by absorption methods prior to ID.

As a test for antibody, ID has obvious potential as an epidemiological tool for serological surveillance. The demonstration of antibody in 100/100 adult bovine sera from diverse sources indicates a sensitivity approaching 1.00 for this test.

It was not determined whether the faecal antibody was of maternal or intestinal origin. Speculatively, the detection of antibody in faeces may be diagnostically useful when more information is available relating its occurrence to that of disease.

While ID is based on reference reactants derived from bovine sources, it appears to be equally applicable to RV infection of other species, including man. It has the practical advantage of readily available reference reactants, simplicity of procedure and facilities required, high specificity and adequate sensitivity.

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