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Some observations on the use of the Ouchterlony gel diffusion technique in the study of myxomatosis

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INTRODUCTION

Several workers have studied the immunological response of the rabbit to myxoma and fibroma viruses. Mansi (1957) and Mansi & Thomas (1958) used the Ouchterlony gel diffusion precipitin technique to study the course of the immunological response. In the latter paper a suggestion was made that it might be possible to differentiate between typical and atypical forms of the disease myxomatosis by the use of the gel test. The present work is an extension of the Mansi & Thomas investigation. This work is in two parts. First, there is a study of laboratory infections of domestic rabbits and this was carried out at the National Institute for Medical Research (N.I.M.R.,) Mill Hill; the Microbiological Research Establishment (M.R.E.), Porton, and the Ministry of Agriculture, Fisheries and Food, Field Research Station, Worplesdon. The second part is concerned with the study of the antigen and antibody in wild rabbit carcasses obtained from one outbreak of the disease.

MATERIALS AND METHODS

The gel diffusion technique as reported by Darbyshire (1962) for the study of mucosal disease of cattle was closely followed. This technique is almost identical with that employed at the Central Veterinary Laboratory for the diagnosis of myxomatosis. The size and distances apart of the wells used in these experiments are shown in Fig. 1*a*.

Antigens

Primary lesion, lung and blood clot (or serum) were used as test material. The tissues were cut up so that they fitted cleanly into the wells prepared in the agar. The control (positive) antigen was lesion homogenate from an infected rabbit.

Antisera

The control (positive) antiserum was a serum pool from rabbits which had recovered from myxomatosis.

Control antiserum and antigen were samples of that used at the Central Veterinary Laboratory.

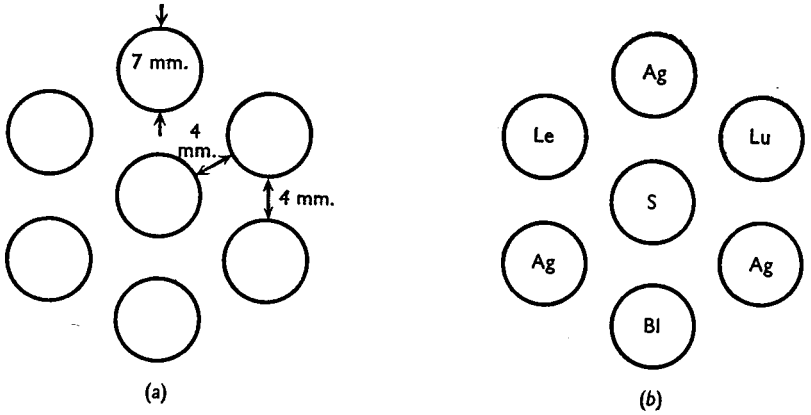

Fig. 1. (*a*) Sizes and distance of wells. (*b*) Arrangement of tissues and controls. Ag, known myxoma antigen (control); S, myxoma antiserum. From animals under test: Le, lesion material; Lu, lung; Bl, blood clot or serum.

Agar

Two agar media were used:

(1) Oxoid Ionagar no. 2 (1·5 %, w/v) in 0·85 % (w/v) sodium chloride buffered with 0·01 M phosphate and with 0·5 % (w/v) phenol to control growth of micro-organisms. The final pH was adjusted to 7·2, using N/10 sodium hydroxide. Plates were prepared by pouring 12 ml. agar medium in 9 cm. diameter Petri dishes. Used at N.I.M.R., Mill Hill.

(2) Oxoid Ionagar no. 2 (1·5 % w/v) in 0·01 M sodium barbitone/hydrochloric acid, buffer pH 7·2. 20 ml. agar was poured into 9 cm. diameter Petri dishes. Used throughout, except at N.I.M.R.

Rabbits

Domestic rabbits weighing 4–4½ lb. were kept in individual cages in rooms which had a constant temperature (Worplesdon, 15·6° C. (60° F.); M.R.E., 18·5° C. (65° F.); N.I.M.R., not recorded).

Wild rabbits were obtained from a large population occupying an area of approximately 200 acres of a Yorkshire dale. One hundred and twenty-four rabbit carcasses were obtained over a period of 3 days; the majority were shot but a few were taken by snaring and dogs. On receipt in the laboratory pieces of eyelid, lung and blood clot were removed from each carcass and stored at −20° C. until required. A record was kept of the weight and sex of each rabbit.

Virus strains. These are listed in Table 1.

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Table 1. *Virus strains*

Name	Synonyms and isolation	Origin	No. of laboratory passages
A & H	Mouse brain adapted (Andrewes & Harisijades, 1955)	Mouse brain adapted	Several
Cornwall	England/Cornwall/4–54/1 (Hudson, 1954, unpublished; Rowe, Mansi & Hudson, 1956)	Diseased wild rabbit	Several
Glenfield	Aust./Dubbo/2–51/1 (see Fenner & Marshall, 1957)	Naturally infected wild rabbit	*c.* 100
Cornwall–Port Looe	TPM 6/62 (Chapple & Lewis, 1962, unpublished)	Diseased wild rabbit	0
Atro (R 244)	England/Sussex/10–54/1 (Andrewes, Muirhead-Thomson & Stevenson, 1956)	From mosquito pool (*Anopheles maculipennis atroparvus*)	Several
Brecon	MX 4298 (Chapple & Bowen, 1963)	Diseased wild rabbit	0
KM 13	Aust./Corowa/12–52/2 (Myers, Marshall & Fenner, 1954)	From pool of *A. annulipes*	1
Cornwall–Tor Point	MX 4333 (Chapple & Lewis, 1962, unpublished)	Diseased wild rabbit	0
Devon—Bere Ferrers	TPM 1/62 (Chapple & Lewis, 1962, unpublished)	Diseased wild rabbit	0
Devon—Bere Ferrers	TPM 2/62 (Chapple & Lewis, 1962, unpublished)	Diseased wild rabbit	0
Devon—Bideford	TPM 3/62 (Chapple & Lewis, 1962, unpublished)	Diseased wild rabbit	0
Devon—Bideford	TMP 4/62 (Chapple & Lewis, 1962, unpublished)	Diseased wild rabbit	0
Cornwall–Tor point	TPM 5/62 (Chapple & Lewis, 1962, unpublished)	Diseased wild rabbit	0
St Austins Dale (Yorks)	TPM 13/62 (Chapple & Lewis, 1962, unpublished	Diseased wild rabbit	0
St Austins Dale (Yorks)	TPM 15/62 (Chapple & Lewis, 1962, unpublished)	Diseased wild rabbit	0
Durham	MX 4297 (Chapple & Bowen, 1963)	Diseased wild rabbit	0
Notts	England/Nottingham/4–55/1 (Hudson, Thompson & Mansi, 1955)	Diseased wild rabbit	?

EXPERIMENTAL PROCEDURE

(*a*) *Laboratory infections*

Rabbits which were considered to be susceptible to myxomatosis were injected intradermally in the shaved right flank, by means of a virus-contaminated needle. The number of rabbits used for each strain is indicated in Table 2. These animals were subsequently kept under observation and the infection was allowed to run its normal course with the exceptions as indicated in Table 2. At death, blood,

Table 2. *Summary of results using laboratory rabbits*

No. of rabbits examined	Virus strain	Virulence (grading according to method of Fenner & Marshall, 1957)	Mean survival time and range in days	Gel test result no. rabbits (with their survival time in days in parentheses)			
				Antigen only	Antigen and antibody	Antibody only	Recoveries
9	A & H	I	14 (10–21)	9 (10, 10,* 10,* 10,* 13, 14, 13, 14, 21)	0	0	0
5	Cornwall	I	1 failed to be infected 14 (13–15)	4 (13, 13, 15, 15)	0	0	0
15	Glenfield	I	12·6 (8–24)	12 (13, 13, 13, 14, 24, 11, 11, 11, 8, 9, 13)	3 (13, 13, 13	0	0
5	TPM 6/62	II	13·5 (10–18)	5 (10, 12, 14, 14, 18)	0	0	0
8	Atro (R244)	III	22 (16–25)	7 (25, 23, 22, 16, 23, 25, 20)		1 (recovery)	1
15	Brecon	III	24 (17–47)	6 (19, 28, 19, 17, 22, 21)	6 (25, 33, 26, 24, 47, 35)	3 (2 recoveries 85†)	3
5	Brecon	III	Killed at 6 days	3‡ (—)	0	0	0
5	KM 13	III	19·3 (16–22)	3 (16, 20, 22)	0	2 (recoveries)	2
5	MX 4333	III	18·4 (10–24)	2 (18, 13)	3 (21, 10, 24)	0	0
5	TPM 1/62	III	21·3 (16–25), 1 failed to be infected	1 (16)	2 (23, 25)	1 (recovery)	1
4	TPM 2/62	III	24 (22–25)	1 (22)	3 (25, 26, 23)	0	0
5	TPM 3/62	III	18·8 (16–27)	4 (16, 16, 17, 18)	1 (27)	0	0
5	TPM 4/62	III	18·2 (15–22)	4 (13, 18, 23, 15)	1 (22)	0	0
5	TPM 5/62	III	18·0 (14–20)	3 (20, 17, 19)	2 (20, 14)	0	0
5	TPM 13/62	III	16·6 (14–21)	3 (12, 21, 21)	2 (14, 15)	0	0
5	TPM 15/62	III	16·2 (11–22)	3 (11, 13, 15)	2 (22, 20)	0	0
5	Durham	IV	2 failed to be infected	0	0	3 (recoveries)	3
4	Notts	IV	2 at 17 days, 1 died at 17 days, 1 failed to be infected	3 (17,* 17,* 17)	0	0	0

* Killed.

† Rabbit considered recovered, death was due to a cause other than myxomatosis.

‡ Two showed no antibody or antigen.

lung and primary lesion material were removed and tested against control (positive) antigen and antiserum.

The control serum was used undiluted; the respective dishes were filled to capacity. The arrangement of tissues and controls was always as in Fig. 1*b*. The plates were either incubated at 22° C. or left on the bench at room temperature. The plates were examined daily for 3 days before the result was finally recorded. Reading was carried out in a darkened room using an intense oblique light source.

(*b*) *Natural infections*

Since the original epizootic of 1953–55 samples of myxomatous rabbits have been sent regularly to the Central Veterinary Laboratory for examination by the gel-diffusion technique. A rabbit carcass was sent from the outbreak now under discussion and material from this rabbit was sent to Worplesdon. Virus was extracted from the material and injected into a group of five rabbits to assess the virulence according to the method originally employed by Fenner & Marshall (1957) and later used in this country by Chapple & Bowen (1963). All the rabbits died within 21 days, giving a mean survival time of 16·6 (range 14–21) days. On the basis of this mean survival time, and the clinical symptoms, the disease was assessed as being moderately severe. It was impossible to distinguish the virus causing this outbreak from that which produced the original epizootic in 1953–55 simply on the basis of the appearance of the rabbits in the field. The rabbit material used in the experiments now described was obtained about 10 weeks after the sample which was used to ascertain the mean survival time. An assessment of the virulence of the virus at this time resulted in a mean survival time of 19 (range 18–21) days.

All the tissues extracted from the rabbit carcasses were put up against known myxoma antigen and the pooled sera of recovered rabbits. On receipt of the rabbit carcasses a visual assessment of the clinical condition was made. The obvious absence of disease and obvious infection were labelled 'Clean' and 'Infected' respectively. The remainder were divided into two groups; first, those that looked reasonably healthy but owing to the condition of the carcass it was not possible to be certain; these were labelled '?Clean'. Secondly, those carcasses which were suspected of being infected but were also in such a condition as to make a positive diagnosis impossible, were designated '?Infected'. The clinical diagnosis is compared with the diagnosis using the gel test, in Table 3.

Figures 2–4 indicate in diagrammatic form some of the possible results in the gel test.

Fig. 2*a* shows the presence of antigen in all the tissues tested. Fig. 2*b* shows the presence of antibody in lung and blood clot; antigen is still present in the lesion material. It is possible to get antibody present in all the tissues tested. A negative reaction (no antibody or antigen detected) is shown by Fig. 4. These are the results which are frequently seen. However, there are occasions when only weak reactions are detected and two of these are illustrated in Figs. 3*a* and *b*. When an animal is killed early in the course of the disease (i.e. before generalization of the virus) only antigen is detectable and this is found exclusively in the site of inoculation.

Table 3. *Comparison of the clinical and gel test diagnosis and the attempted differentiation of virus by means of the gel test*

(Figures indicate numbers of rabbits.)

	Gel test diagnosis				
	Infected				
Clinical condition	Typical	Atypical	Total	Immune	Susceptible
Clean	4	10	14	1	20
Infected	23	55	78	1	1
? Infected	1	2	3	0	1
? Clean	0	1	1	0	2
Unknown	1	1	2	0	0
Total	29	69	98	2	24

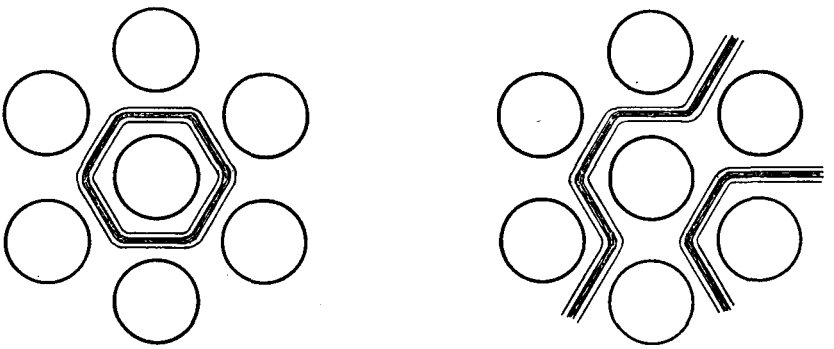

Fig. 2. (*a*) One of the possible gel results which would lead to a diagnosis of 'typical' (antigen only detected). (*b*) One of the possible gel results which would lead to a diagnosis of 'atypical' (antigen and antibody detected in the test materials).

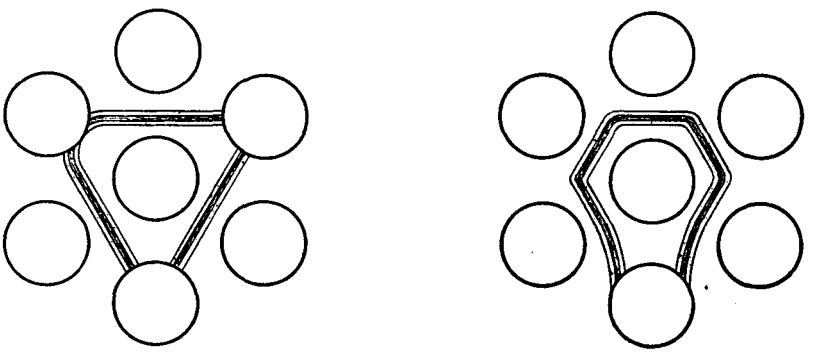

Fig. 3. (*a*) Weak 'antigen' reaction with lesion material. (*b*) Weak antibody reaction from blood.

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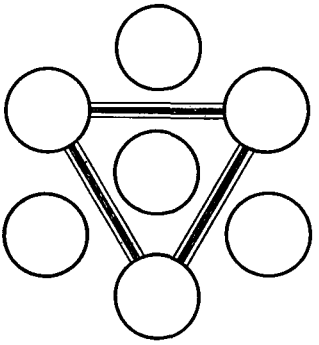

Fig. 4. Negative reaction (no antibody or antigen detected in the test materials).

RESULTS

The results of the gel tests in laboratory rabbits together with the mean survival times and the virulence classification of the virus strains are summarized in Table 2.

The wild-rabbit carcasses which were diagnosed as infected on the basis of the gel test results were typed according to the tentative hypothesis of Mansi & Thomas (1958). This hypothesis is summarized below.

Typical—only antigen detected in any or all of the material tested.

Atypical—any instance in which antibody is found with or without the presence of antigens except where antibody is detected only in the blood.

Immune—antibody detected only in the blood.

Susceptible—no antigen or antibody detected.

The effect of applying this to the results obtained with the sample of rabbits now under discussion is also shown in Table 3.

DISCUSSION

Mansi (1957) and Mansi & Thomas (1958) showed that myxoma and fibroma viruses could be identified by means of a modified Ouchterlony test in agar gel. Subsequently this test has been used for the routine diagnosis of myxomatosis at the Central Veterinary Laboratory, Weybridge. On the basis of the pattern produced by the precipitation lines with certain strains of myxoma virus Mansi & Thomas (1958) suggested, in their discussion, that it might be possible to differentiate between what they referred to as 'typical' and 'atypical' forms of the disease. Unfortunately the terms typical and atypical have been used, by some people, as if they were synonymous with the disease caused by virulent and attenuated forms of virus respectively although nothing has been published which would lead to such a conclusion.

However, it should be remembered that the hypothesis stems mainly from the work reported by Mansi & Thomas (1958) with two field strains of virus; the fully virulent 'Cornwall' (Rowe, Mansi & Hudson, 1956) and the supposedly attenuated 'Nottingham' strain (Hudson & Mansi, 1955; Fenner & Marshall, 1955). These

two strains were taken as representative of those causing 'typical' and 'atypical' infections respectively. The latter strain (Nottingham) has been shown to be a mixture of two strains—one fully virulent and the other a markedly attenuated strain (Fenner & Marshall, 1957).

In the present work it was thought advisable to examine several strains of each major type (fully virulent and attenuated). The relevant experimental results are summarized in Table 2.

As a working hypothesis it was assumed that all fully virulent strains of virus would yield the same result and that all attenuated strains would yield the same result. The experiments recorded in this paper indicate that this was too broad a generalization. Out of fifteen rabbits infected with the Glenfield strain of virus (a fully virulent Australian strain used for initiating epizootics) nine behaved in the expected manner. However, three rabbits gave a picture which would have definitely resulted in a report of atypical being given, i.e. antibody was detected as well as antigen. It is significant that these three rabbits were not ones which had a longer than normal survival time as one might have expected if the Mansi & Thomas hypothesis was correct. On the contrary one rabbit which was classified as 'typical' in fact survived for 24 days which is certainly unusual for this strain (F. Fenner, personal communication). The fact that antibody is not detected in the gel test does not necessarily mean that the strain of virus concerned is 'typical' fully virulent. An examination of Table 2 shows that of the rabbits inoculated with attenuated strains of virus, seventy-five died or recovered. There were ten recoveries (including the rabbit which died at 85 days) and of the remainder twenty-two showed antibody and antigen giving a gel diagnosis of atypical, and forty-three showed antigen only, giving a gel diagnosis of typical. However, because the strains of virus which caused the disease were known to be attenuated it would seem that the postulate of Mansi & Thomas (1958) does not always hold good and that it appears on the basis of these laboratory infection experiments that interpretation of the results of gel diffusion tests must be made with great caution.

Because of the suggestion that there might be significantly longer mean survival times obtained from wild rabbits, when compared with the domestic form (Fenner & Marshall, 1957) it was considered necessary to assess the gel test using a large population of wild rabbits. It was not possible to obtain a large number of live wild rabbits for use in the laboratory, nor to predict effects on the course of the disease of keeping live wild rabbits in captivity. Therefore, the rabbits studied were obtained from a large natural population which had had myxomatosis present for about 10 weeks. The sample was obtained over a period of 3 days. It is not suggested that the sample obtained (124 rabbit carcasses) was random but it was considered sufficiently large to include a number of normal and possibly recovered rabbits.

The first point to be examined was whether the gel test could provide information purely on the presence or absence of disease, over and above the information obtained by inspection of a carcass. The second point was whether the tentative suggestion of Mansi & Thomas (1958), that the gel test could differentiate between typical and atypical forms of disease, was tenable in a wild rabbit population.

An examination of Table 3 shows that of eighty rabbits which were obviously infected, seventy-eight gave a positive gel test confirming infection. In one of the remaining two, antibody only was detected (in the blood) giving a gel diagnosis of recovered. The second carcass did not show antibody or antigen in the materials tested. It is known that rabbits in the early stages of recovery often show marked nodular lesions and these would lead to a clinical diagnosis of infected. Conversely, it is possible, very occasionally, to pick pieces of tissue from a carcass which do not contain antigen or antibody in demonstrable amounts and this could explain the infected carcass which was typed as susceptible. These results are quite satisfactory but when one examines those obtained from rabbits which were called clean (after clinical inspection) then the situation is not satisfactory. Thirty-five rabbits were described as clean and of these fourteen were found to be infected, one was typed as recovered and twenty were typed as susceptible as a result of using the gel test. However, a closer examination of the fourteen rabbits typed as infected shows that only five had detectable antigen and the remaining nine had antibody only. From this it would be safe to assume that these nine rabbits were recovering or had recovered from the disease.

In the remaining three groups, ?Infected, ?Clean and Unknown, there are insufficient numbers to reach any definite conclusion. However there is the suggestion that the provisional clinical diagnoses were justified.

Table 4 is similar to the top line of Table 3, but makes use of a revised definition of a recovered (≡ immune—Mansi & Thomas, 1958) rabbit. It is suggested that this is a rabbit which is clinically clean and shows antibody alone in any or all of the tissues examined. Using this revised definition only five rabbits were classified as infected; of these, four showed antigen only, thus suggesting that they were in the early stages of the disease.

Table 4. *Comparison of clinical and gel test diagnosis using the revised definition of a recovered rabbit*

Clinical diagnosis	Gel test diagnosis			
	Infected	Recovered	Susceptible	Total
Clean (1)	14	1	20	35
Clean (2)	5	10	20	35

Clean (1): gel test interpretation as in Table 3. Clean (2): gel test interpretation revised.

The outbreak of myxomatosis under discussion was caused by a single virus introduction and we have reason to believe that no further introductions were made. The results are summarized in Table 3. The definitions for the gel test categories are those which are in current use at the Central Veterinary Laboratory and which have been described previously. From these results it would appear that there are two virus strains present, a fully virulent and an attenuated strain. As a result of our knowledge of the history of the outbreak, together with laboratory assessment of virulence, we have concluded that there was only one virus

strain involved. Therefore, the apparent differentiation shown by the gel test results was not valid. The course of the outbreak of the disease was much slower than that associated with disease caused by fully virulent virus. This slowness might have been caused by lack of suitable vectors. However, flea counts on rabbits sent in to the laboratory since the start of the outbreak suggest that there are adequate numbers of vectors. Of twenty rabbits examined we found from three to thirty-six rabbit fleas (*Spilopsyllus cuniculi*) present on each rabbit and the average was nineteen. Mead-Briggs (1963) has shown that the rabbit flea is a more mobile vector than was hitherto supposed. Thus any slowness in the spread of the disease within the population was probably due to the nature of the virus rather than the vector.

We have concluded that the gel test can provide valuable information on the infection and the recovery rate of wild rabbit populations provided that the clinical diagnosis is taken into consideration. However, the results show that it is impossible to differentiate fully virulent from attenuated virus using this technique.

SUMMARY

1. Laboratory rabbits were infected with seventeen different preparations of myxoma virus (this probably represents thirteen strains of which four could be classified as fully virulent and the rest as attenuated, two being markedly so).

2. One hundred and twenty-four wild rabbit carcasses were obtained from a rabbit infestation in the throes of an outbreak of myxomatosis.

3. All the carcasses were examined for the presence or absence of myxoma antigens and antibodies by a modified Ouchterlony gel-diffusion precipitin test.

4. As a result of (3), we have concluded that the gel test can provide confirmation of the presence or absence of myxomatosis. It is particularly useful in cases of doubt about the clinical diagnosis. The test can also give a reasonable indication of the number of recovered rabbits in a population and, more important still, it can give an indication of the recovery rate during an outbreak of disease. However, attempts to differentiate between fully virulent and attenuated strains of virus were unsuccessful.

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