SHORT ARTICLES

STANDARDISATION OF INOCULA FOR ASSESSING THE VIRULENCE OF STRAINS OF *AVIUM-INTRACELLULARE* MYCOBACTERIA IN CHICKENS

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COMPARATIVE studies on the virulence and pathogenicity for chickens of mycobacterial strains within the *Avium-Intracellulare* complex have been carried out at this Institute. When the challenge dose was based on the wet weight of organisms, inconclusive and variable results were obtained (unpublished observations). This paper describes a method for preparing inocula containing known numbers of viable organisms, for use in assessing the virulence of strains for chickens.

MATERIALS AND METHODS

Medium. The medium used for the cultivation of mycobacteria was similar to that described by Stuart (1965). To 96 ml of Stuart's basal medium were added 4 ml of 9% (w/v) bovine albumin (Armour Laboratories) and crude liquid mycobactin to give a final concentration of 3.0 µg per ml.

Bacterial strains. The strains used were taken from the collection isolated from pigs by Matthews (1969). Strain A was subcultured several times before being passaged once through chickens. The re-isolated strain was stored at -70°C as our standard *Mycobacterium avium* strain. All other strains had undergone subculture; they too were stored at -70°C . Only strain D was found to be mycobactin-dependent at the time of isolation.

Preparation of challenge doses. The growth from a mature culture was harvested in 5 ml isotonic saline in a weighed bottle. After centrifugation at 800 *g* for 15 min. the supernatant fluid was removed and the bottle reweighed. The wet bacteria were then resuspended to a concentration of 10 mg per ml in Stuart's liquid medium without mycobactin. The suspension was distributed in 1.0-ml aliquots and stored at -70°C . Experiments previously carried out to determine loss of viability during storage at -70°C in Stuart's liquid medium indicated that, apart from occasional loss at the time of freezing, the viable counts remained unchanged over a period of 1 year.

Viable counts were made by the method of Miles, Misra and Irwin (1938) in 5-cm Petri plates containing Stuart's medium with mycobactin. The plates were sealed and incubated at 37°C . A stereoscopic microscope was used to count the mature colonies that had grown after 14–21 days. Viable counts of bacterial suspensions were made on at least two occasions during storage. At the time of challenge the suspensions were if necessary adjusted to contain 1000–2500 million organisms. Ten-fold dilutions were then injected intravenously in 1.0-ml doses into chickens as shown in the tables. Viable counts were carried out immediately before inoculation to confirm retrospectively the numbers of organisms injected. If any inoculation was attended by a suspicion of perivascular leakage, the suspicion was recorded.

At necropsy, viable counts were made of the mycobacteria in the liver and spleen. A 10% (w/v) homogenate in 5% (w/v) oxalic acid was prepared from each tissue in a Griffith's grinding tube. After incubation in a waterbath at 37°C for 45 min., ten-fold dilutions

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were made. Standard drops (0.02 ml) were then placed on Stuart's medium containing mycobactin.

RESULTS

Viable counts of suspensions standardised on the basis of wet weight

Viable counts of suspensions (10 mg per ml) of six strains belonging to the *Avium-Intracellulare* group varied considerably. The counts obtained were (millions per ml) 280, 650, 1470, 4600, 6700 and 21 400.

Inoculation of chickens with known numbers of viable units

Table I shows the combined results of four separate experiments in which strain A was injected intravenously into chickens in pairs or small groups. Table II shows the combined results of two further experiments in which strains B, C and D were used.

The results demonstrate the success of the technical methods in preparing challenge doses of the required size; for each strain used the pathogenic effects were, within the limits of individual variation, dose related.

Viable counts from liver and spleen

The viable counts from chickens that died after experimental infection with strain A are shown in table III. Counts of <1.0–2000 million per g were obtained and the spleen usually contained more viable units than the liver. Similar results were obtained from the liver and spleen of the chickens inoculated with strains B, C and D.

TABLE I

Mean survival times of chickens inoculated intravenously in four experiments with graded doses of viable units of M. avium, strain A

Experiment number	Number of		Survival time (days)	
	viable units injected	chickens	Mean	Range
2	1.4×10^7	2	36	} 28–40
3	1.7×10^7	2	30	
1	5.0×10^6	2	32	} 32–42
2	1.4×10^6	2	36	
3	1.7×10^6	2	41	
1	5.0×10^5	2	54	} 53–81
2	1.4×10^5	2	64	
3	1.7×10^5	2	71	
4	1.65×10^5	5	66	
1	5.0×10^4	2	71	} 60–119
3	1.7×10^4	2*	76	
4	1.65×10^4	10	106	
4	1.65×10^3	10	129	102–162

* Perivascular leakage suspected in one bird at the time of inoculation; the bird was killed on the 126th day and disregarded for the purpose of calculating means and ranges of survival times.

TABLE II

Survival times of chickens inoculated intravenously in two experiments with graded doses of three strains belonging to the Avium-Intracellulare group

Strain	Experiment number	Number of		Survival times (days)
		viable units injected	chickens	
B	5	2.5×10^6	2	49, 49
B	6	1.7×10^6	1	86* (died)
C	5	2.2×10^6	2	53, 64
C	6	2.1×10^6	1	62
D	5	1.8×10^6	2	39, 54
D	6	1.8×10^6	1	52
B	5	2.5×10^5	2	89, 130*
B	6	1.7×10^5	2	82, 112
C	5	2.2×10^5	2	58, 71
C	6	2.1×10^5	2	42, 61
D	5	1.8×10^5	2	66, 68
D	6	1.8×10^5	2	58, 65
B	5	2.5×10^4	2	118, 124
B	6	1.7×10^4	2	105, 130*
C	5	2.2×10^4	2	74, 92
C	6	2.1×10^4	2	65, 112
D	5	1.8×10^4	2	83, 108
D	6	1.8×10^4	2	87, 102

* Perivascular leakage suspected at the time of inoculation; bird killed on the day indicated unless otherwise stated.

DISCUSSION

Griffith (1911) carried out a comprehensive study of different methods of exposure to doses of *M. avium* varying from 50 to 0.0000001 mg. Doses of 1.0 mg given intravenously killed chickens in about 33 days; larger doses of 10–50 mg did not cause the chickens to die any earlier. Chickens inoculated with extremely low doses all showed signs of tuberculosis when killed at the 94th day.

To determine the virulence and pathogenic effect of different strains of mycobacteria, and to measure any benefit from immunological procedures, it is necessary to challenge with doses of a suitable size. It is usually accepted that 0.1 mg of *M. avium* cells given intravenously will kill chickens within 8–12 weeks. Our observations on suspensions (10 mg per ml) of six strains showed that a 0.1-mg dose would have contained (millions) 2.8–214 viable units.

TABLE III

Viable counts of organisms in the livers and spleens of chickens that died after inoculation with graded doses of M. avium, strain A

Chicken number	Dose of viable units	Number of viable units (millions) per gram of		Time of survival (days)
		liver	spleen	
1	1.4×10^5 {	85	N	61
2		12	5	67
3	1.7×10^5 {	920	20	71
4		6	775	72
5	1.65×10^5 {	250	1270	81
6		170	500	56
7		250	220	64
8		170	970	70
9	1.7×10^4 {	32	15	76
10		<1	<1	126*
11	1.65×10^4 {	20	87	75
12		2000	1500	111
13		65	742	105
14		150	1500	85
15		2	90	105
16		30	600	103
17	1.65×10^3 {	270	260	148
18		20	25	128
19		150	17	156
20		7	220	127
21		47	240	133
22		300	370	159
23		5	5	102
24		170	510	137

N = Not done.

* Perivascular leakage suspected at the time of inoculation; bird killed on the day indicated.

To compare the virulence of strains within the *Avium-Intracellulare* complex it is important to inject inocula containing known numbers of viable units, because viability may not be correlated with mass.

In a study of *M. avium* strains isolated from wild birds, Schaeffer *et al.* (1973) illustrated the limitations of virulence tests when there was a tendency for the strains to be rough.

We have confirmed the findings of Kim and Kubica (1972) that storage at -70°C is a suitable method for maintaining the viability of *M. avium* suspensions destined for inoculation in accurately standardised doses.

Many of the strains under study at this Institute were mycobactin-dependent at primary isolation and medium containing mycobactin has therefore always been used; in our experience this results in improved growth and smoothness of colonies both at primary isolation and on subculture. Nevertheless, many of the strains are rough and this results in low viability in terms of wet-weight. It is doubtful whether 0.01 mg of a rough strain would be sufficient to cause infection and death in chickens because, in our experience, such a dose would contain only a few hundred viable units.

The observations of Griffith (1911) and the results obtained with strain A (table I) indicate the range of doses likely to be useful for measuring virulence. From the mortality produced by doses of 1.65×10^4 to 5.0×10^5 viable units, it would seem that in this range an approximately ten-fold increase in dose may be expected to produce a decrease of approximately 4 weeks in survival time. Towards the upper end of the dose range, the survival times differed in duration by not more than about 30 days. This figure increased to 60 days when the dose was reduced to 1.65×10^3 .

Generally similar results were obtained with the three further strains B, C and D; it is of interest that strain D, although mycobactin dependent on primary isolation, had a virulence for chickens similar to that of strains A, B and C.

Although the viable counts in the liver and spleen of dead birds frequently exceeded 10^8 per g, the results suggested that once a peak of 10^6 – 10^8 viable units per g had been reached, death resulted; the smaller the inoculum, the longer it took to reach this level. Chicken no. 10, a bird that may not have received the full inoculum intravenously due to perivascular leakage, when killed at 126 days had a viable count of $<10^6$ viable units per g in both liver and spleen and may well have survived a good deal longer before the peak was reached. It is possible that if chickens were inoculated with low doses and killed at a suitable time, differences in virulence might be demonstrated by the numbers of viable organisms present.

The livers and spleens from chickens that survived longer than 12 weeks often showed tuberculous nodules up to 8 mm in diameter, with early calcification; this tended to lower the viable counts.

A study of the virulence of different strains and serotypes within the *Avium-Intracellulare* group by means of methods such as those described might be of taxonomic value.

SUMMARY

The number of viable units present in wet-weight suspensions of mycobacteria varied considerably. To compare the virulence of strains within the *Avium-Intracellulare* complex, standard inocula containing known numbers of viable units are therefore recommended. The preparation of standardised inocula and their use in virulence studies are described.

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