GROWTH, SURVIVAL, ANTIGENIC STABILITY, AND VIRULENCE OF *LEPTOSPIRA INTERROGANS* SERO-TYPE *CANICOLA*

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VACCINES prepared against *Leptospira interrogans* serotypes *canicola*, *icterohaemorrhagiae* and *pomona* are widely used for the immunisation of domesticated animals in the USA, and vaccines against serotypes *hardjo*, *grippotyphosa* and *szwajizak* are at present under consideration. Both initial licensing and periodic potency testing of licensed leptospiral vaccines in the USA depend upon testing in hamsters.

Data related to the in-vitro preservation of leptospire has been published recently (Ellinghausen, 1973*a* and *b*). The present paper is concerned with serotype *canicola* and with matters of practical importance in relation to the propagation and testing of leptospiral vaccines. The scope of the experiments encompassed (1) investigation of the growth of virulent organisms in liquid, semisolid, and solid "polysorbate media", or in the same media after certain nutrient constituents had been either omitted or replaced by others, (2) assessment of the effect of simplified semisolid media other than "polysorbate media" on growth and on the preservation of virulence, (3) antigenic stability in different culture media, and (4) virulence after storage in culture media, suspending fluids and kidney homogenates, and virulence of cultures grown from single colonies.

MATERIALS AND METHODS

Media. The standard "polysorbate medium" contained polysorbate 80 (Tween 80), bovine albumin, ammonium chloride, thiamine, vitamin B₁₂, sodium chloride, magnesium chloride, trace metals, and l-cystine, and it is referred to in this paper as P-80 medium and considered a complete medium. Media in which polysorbate 80 only was deleted and replaced by polysorbate 60, 40, or 20 are referred to respectively as P-60, P-40, and P-20 media. The media, already fully described by Ellinghausen (1973*a* and *b*), were used in the liquid, semisolid (0.2% agar) and solid (1% agar) forms.

Further modified media were produced by omitting certain constituents or replacing them by others. Eight such media shown in table I were employed to define better the multiple role that albumin plays. The media so listed in table I will hereafter be referred to as single or multiple deletion media. The "single deletion" media were complete in all respects except for the individual deletion of polysorbate 80, ammonium chloride,

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thiamine, or vitamin B₁₂. The "multiple deletion" media lacked both polysorbate 80 and ammonium chloride, and sometimes also one or both of thiamine and vitamin B₁₂.

Asbestos-filtered P-80 medium was complete except for any substances removed as a result of the method of sterilisation.

"Simplified" semisolid media were made with a base of 0.005M phosphate (KH₂PO₄ 87 mg per l and Na₂HPO₄ 664 mg per l) partially solidified with 0.2% agar. This agar-phosphate basal medium was used unsupplemented or supplemented with either (1) 1% bovine albumin, (2) 1% albumin and vitamin B₁₂, (3) 1% albumin and thiamine, or (4) 1% albumin, vitamin B₁₂ and thiamine. The simplified media lacked the NH₄Cl, MgCl₂, NaCl, trace metals, l-cystine, and polysorbate present in complete P-80 semisolid medium.

Leptospiral growth measurement and microscopic agglutination (MA) test. These have already been described by Ellinghausen (1973a, b, and c).

Leptospira interrogans serotype canicola. Except where otherwise stated, the strain used was the virulent strain A-13 of the National Animal Disease Center culture collection (NADL A-13), maintained in P-80 semisolid medium at 23–25°C for 5 years.

Antigenic stability. After growth in various media, *canicola* was tested by the MA method with antisera against *canicola* (NADL A-13), *canicola* (Hond Utrecht IV), *icterohaemorrhagiae* (RGA), *copenhageni* (M-20), *dakota* (Grand River) and *ballum* (S-102). These strains were chosen because they represent leptospires that typically cross-agglutinate in various patterns with *canicola*. Serotype *canicola* antigen for the MA test, standardised as described by Ellinghausen (1973a), had a nephelometer reading of 25 and a Petroff-Hauser chamber count of 260×10^6 per ml.

Inocula for culture media and experimental hamsters. One hundred cultures of strain *canicola* (NADL A-13) in semisolid P-80 medium were stored at 23–25°C for future use. Two millilitres of preserved culture were gently inoculated drop by drop on to the surface of 10 ml of fresh P-80 semisolid medium and incubated at 29°C until well grown. All such medium was tested before use to ensure that 10 ml was capable of producing growth from an inoculum of two organisms. Two millilitres of the fresh culture were transferred to 10 ml of liquid P-80 medium, vigorously shaken, and incubated for not more than 72 h. To ensure the absence of more than a trace of agar, one further subculture was made in liquid P-80 medium and incubated until turbidity measurements indicated the required degree of growth.

Continuous subculture. One-ml volumes of liquid culture were transferred to 10-ml volumes of parent medium and, after incubation, the process was repeated. All subcultures were incubated at 29°C for 14 days except those in complete P-80, P-60, P-40, and P-20 media, which were incubated for 7 days only.

Virulence tests in hamsters. Virulence titrations were made in weanling hamsters given intraperitoneally 1-ml amounts of selected serial 10-fold dilutions of bacterial suspension. The original suspension had a nephelometer reading of 25 and a Petroff-Hauser count of 260×10^6 cells per ml. The doses selected were 26×10^6 , 2.6×10^3 , 2.6×10^2 , 26, and 2 leptospires per animal and all dilutions were made in 1% bovine-serum-albumin diluent (BSAD) (Ellinghausen, 1973a).

Every experiment recorded in this paper was preceded by a confirmatory test of virulence; the test consisted of inoculating five weanling hamsters intraperitoneally with 2.6×10^3 leptospires and observing the characteristic mortality pattern together with the distribution of organisms in the brain, liver, kidney and blood at the time of death or slaughter.

RESULTS

Growth, viability, and continuous subculture in 13 liquid media

All the media contained 1% albumin. Inocula that supplied as few as two or three bacterial cells produced growth in P-80, P-60, P-40, and P-20 media and in P-80 medium from which either polysorbate 80, NH₄Cl, or

TABLE I
Growth of *Leptospira interrogans* serotype *canicola* (strain NADL A-13)
in 13 liquid media

Medium	Constituents omitted from medium	Nephelometer readings after 21 days' incubation at 29°C of tubes containing medium inoculated with the following numbers of <i>canicola</i> cells per ml							
		26×10^5	26×10^4	26×10^3	26×10^2	260	26	2	0
P-80	None	44	45	51	50	49	22 (52)	6 (50)	0
P-60	None	37	30	44	40	10 (46)	2 (50)	0 (39)	0 (36)
P-40	None	33	36	39	36	36	11 (41)	0 (44)	0 (44)
P-20	None	33	35	39	19 (36)	11 (43)	3 (48)	0 (28)	0 (18)
P-80	Polysorbate 80	10	10	8	8	8	8	6	0*
P-80	NH ₄ Cl	9	10	10	10	10	11	17	15
P-80	Thiamine	10	11	12	10	17	17	16	2*
P-80	Vitamin B ₁₂	49	45	0*	0*	0*	0*	0*	0*
P-80	Polysorbate 80, NH ₄ Cl	9	8	7	9	11	3	0*	0*
P-80	Polysorbate 80, NH ₄ Cl, thiamine	3	2	2	2	2	2	0*	0*
P-80	Polysorbate 80, NH ₄ Cl, vitamin B ₁₂	8	6	0*	0*	0*	0*	0*	0*
P-80	Polysorbate 80, NH ₄ Cl, thiamine, vitamin B ₁₂	3	2	1	1	1	0*	0	0
P-80†	None	42	47	0	0	0	0	0	0

Nephelometer readings after 30 days' incubation are given in parentheses.

* After 21 days' incubation, presence of viable organisms shown by subculturing 1 ml in P-80 semisolid medium. Zero readings without asterisk indicate that tubes were sterile after 21 days' incubation.

† Medium sterilised by a Seitz asbestos filter.

thiamine had been omitted (table I). The omission of vitamin B₁₂ from P-80 medium greatly increased the size of the inoculum necessary to produce growth. The omission of the main carbon and nitrogen sources (polysorbate 80 and NH₄Cl) did not prevent the multiplication of organisms from small inocula, though the degree of growth was greatly reduced. The omission of the four constituents polysorbate 80, NH₄Cl, vitamin B₁₂, and thiamine seriously impaired the growth of *canicola*. Complete P-80 medium sterilised by means of a Seitz asbestos filter was capable of producing good growth, but only from large inocula. Table I also gives information on the survival of *canicola* in tubes that showed little or no turbidity after 21 days' incubation at 29°C.

Continuous subculture of *canicola* was readily possible in P-80, P-60, P-40 and P-20 media, all cultures having a nephelometer reading of 55 (equivalent to approximately 5×10^8 cells per ml). Subculture in P-80 medium lacking polysorbate eventually failed, consecutive nephelometer readings being 9, 7, 6, 3, 2, 3, and 0. P-80 medium without NH₄Cl maintained a constant though low level of growth, the nephelometer readings of seven subcultures being 13, 11, 11, 11, 12, 12, and 12. Thiamine-deleted P-80 medium produced consecutive

subcultures with readings of 58, 38, 6, 1, and 0. Within the space of only three subcultures in vitamin B₁₂-deleted medium, the reading had fallen to 0. P-80 medium lacking both polysorbate 80 and NH₄Cl produced a nephelometer reading of 10 throughout seven subcultures. Continuous subculture proved possible in medium sterilised by means of an asbestos filter if large inocula were used.

Growth in semisolid polysorbate media

Semisolid P-80, P-60, P-40, and P-20 media, and P-80 medium lacking NH₄Cl, all gave growth from an inoculum of only two organisms. A similar inoculum did not produce a well-defined zone of growth (Dinger's zone) in P-80 medium lacking polysorbate 80, although darkfield microscopy demonstrated growth after 21 days' incubation at 29°C. The deletion of thiamine from P-80 medium resulted in growth with a very indistinct Dinger's zone from an inoculum of two organisms, and the leptospire were all of reduced motility. Dinger's zones developed in P-80 medium lacking vitamin B₁₂ from inocula of 26,000 leptospire or more, but not from 2600.

Growth in "simplified" semisolid media

A characteristic Dinger's zone of growth was invariably absent, and darkfield examination was necessary to detect organisms, after incubation at 29°C for 21 days. Unsupplemented medium and medium supplemented with albumin alone or albumin and thiamine were positive on examination by darkfield microscopy when the inoculum was 2.6×10^4 cells, but not when it was 2.6×10^3 cells. Supplementation with both albumin and vitamin B₁₂, either alone or with the addition of thiamine, permitted growth from an inoculum of two cells.

Growth on solid polysorbate media

Table II shows that the various polysorbate media all produced colonies from small numbers of organisms with the exception of P-80 medium lacking vitamin B₁₂.

Antigenic stability during growth in various media

Agglutinating suspensions of strain *canicola* (NADL A-13) were prepared from cultures grown in the first nine of the 13 liquid media already listed in table I. In the MA tests that were then carried out, each of the nine standardised suspensions (nephelometer reading of 25) demonstrated the full titre (10^5) of a homologous antiserum.

After 10 serial subcultures in P-80, P-60, P-40, and P-20 media, *canicola* (NADL A-13) gave typical cross agglutination with antisera against strains *canicola* (Hond Utrecht IV), *icterohaemorrhagiae* (RGA), *copenhageni* (M-20), *dakota* (Grand River), and *ballum* (S-102). Cross-agglutination absorption studies were not performed.

TABLE II
Colony formation by *canicola* (strain NADL A-13) on eight solid media

Medium	Component omitted from medium	Numbers of colonies that grew from the following numbers of organisms			
		5.2×10^6	52	5	0
P-80	None	C	10	1	0
P-60	None	C	C	8	0
P-40	None	C	C	1	0
P-80	Polysorbate 80	C	C	2	1
P-80	NH ₄ Cl	C	37	1	0
P-80	Thiamine	C	35	4	0
P-80	Vitamin B ₁₂	C*	0	0	0

Inocula in 0.2-ml volumes were spread over the plates.

C = Confluent growth in the subsurface of the agar.

* = Growth confluent, but so slight that it was barely detectable.

Virulence tests in hamsters

A typical virulence titration of strain *canicola* (NADL A-13) is shown in table III. Replicate experiments showed that the mean time to death was always predictable as long as the challenge cultures were prepared according to the method described under *Materials and methods*. Bacteriological examination of hamsters that died from the infection and survivors killed 7 days after the last occurrence of death in hamsters that received the smallest dose showed that kidney cultures provided a reliable indication of infection, but cultures from liver, lung, blood, and brain were also frequently positive. Positive tissue homogenates usually yielded growth when diluted 1 in 10^5 , and some were still positive when diluted 1 in 10^8 . Death was sometimes sudden with few premonitory signs, though there was often bleeding from the nostrils and the anus.

The kidneys were always greatly enlarged and engorged with blood. Petechial haemorrhages in the intestinal tract almost always occurred in animals that died. The livers were friable and the lungs were always covered with haemorrhages though to variable degrees.

Survival in BSAD at 23–25°C of organisms derived from infected kidney

Infected kidney tissue was obtained from hamsters that died or became moribund within 5 days of intraperitoneal inoculation with 2.6×10^8 *canicola* cells. A 10% tissue homogenate in BSAD was diluted decimally to extinction in the same medium, the final dilution being 1 in 10^7 . All tubes were kept at 23–25°C and subcultured periodically by transferring 1-ml volumes to semisolid P-80 medium. By this method, viable organisms could be detected up to the

TABLE III

Intraperitoneal virulence titration of canicola (strain NADL A-13) in weanling hamsters

Number of leptospire injected	Number of deaths in groups of 20 hamsters	Mean time (days) to death	Number of survivors* found infected	Positive cultures from various tissues†				
				Kidney	Liver	Lung	Brain	Heart blood
26×10^6	20	4	—	18/20	12/15	5/6	10/10	5/5
2,600	19	9	1	18/19	12/14	11/13	9/10	6/6
260	16	10	4	18/20	18/20	6/7	8/8	6/6
26	13	11	6	18/20	13/20	2/2	7/9	3/3
2	8	13	2	10/20	4/14	4/4	4/6	3/4

* Survivors killed 7 days after the last occurrence of deaths in hamsters that received the smallest dose.

† Numerator = number of positive cultures; denominator = number of hamsters examined.

60th day, but by the 90th day recovery of organisms from the higher dilutions was irregular.

Virulence of canicola after storage

The infected kidney homogenate referred to above was used after 3 and 16 days' storage at 23–25°C to inoculate two tubes of semisolid P-80 medium. Upon appearance of growth, subcultures were made in P-80 liquid medium. A second subculture in P-80 liquid medium was made to rid the culture of traces of agar. The culture originating from the kidney homogenate stored for 3 days was titrated intraperitoneally in graded 10-fold doses ranging from 2.3×10^3 to two organisms in pairs of hamsters. The culture originating from the kidney homogenate stored for 16 days was used to inoculate pairs of hamsters with 2×10^7 , 2×10^3 , 2×10^2 , 20 and 2 organisms. The mortality pattern corresponded closely with that shown in table III.

Organisms grown in artificial culture were suspended in BSAD and in phosphate buffer (PB) (Ellinghausen, 1973a) and kept for 7 days at 23–25°C. Subcultures from the BSAD suspension and PB suspension were each titrated intraperitoneally in graded 10-fold doses ranging from approximately 1.6×10^7 to 1.6 organisms in pairs of hamsters. All died after intervals that could have been predicted from table III.

In a further experiment, the *canicola* strain was stored at 23–25°C in semi-solid forms of the first eight media listed in table I and in each of the five "simplified media" described under *Materials and methods*. The number of organisms per ml inoculated into 10-ml volumes of these 13 semisolid media at the beginning of the storage period was 26×10^6 cells, and 15 months later viable organisms were demonstrated in each of the 13 media by means of subculturing 2 ml in parent medium or P-80 semisolid medium; Dinger's zones appeared in 6–10 days. Four hamsters were given liquid P-80 culture derived from each of the 13 stored cultures. The dose for each hamster was 260 cells,

and there was no evidence that loss of virulence had occurred in the various semisolid storage media. Of the 52 hamsters given inoculations, 37 died in 10 to 11 days and *canicola* was invariably recovered from brain, kidney, and liver. Leptospire were usually isolated from one or more of these organs when the 15 survivors were killed and examined 14 days after infection.

Virulence of cloned colonies

Four, one, one, one, and five single colonies were picked from the following solid media, respectively: P-80, P-60, P-40, P-80 without NH_4Cl and P-80 without thiamine. Each colony was transferred to a semisolid form of the parent medium, then incubated at 29°C for 14 days and subsequently stored at $23\text{--}25^\circ\text{C}$ for 10 months. Each stored culture was then subcultured, first in P-80 semisolid medium and then in P-80 liquid medium, and injected intraperitoneally into between one and four hamsters in doses of 2.6×10^7 and 2.6×10^3 . Of 39 hamsters given inoculations, 32 died from leptospirosis within 10 days, suggesting that little or no loss of virulence had occurred.

DISCUSSION

The fact that P-80, P-60, P-40, and P-20 media all gave satisfactory growth of strain *canicola* (NADL A-13) points to the vigorous growth capabilities of this strain. Some leptospire are much more demanding; for example, certain strains of serotype *hardjo* grow on P-80 medium but are incapable of growth on P-60, P-40, and P-20 medium (Ellinghausen, unpublished observation).

Continuous subculture was possible in P-80 medium from which either NH_4Cl or polysorbate 80 was deleted, provided that both thiamine and vitamin B_{12} were present, and it is felt that the importance of the albumin content of the medium cannot be overstressed. Even in simplified semisolid medium supplemented with albumin, thiamine and vitamin B_{12} , growth occurred; solid medium demonstrated more adequately the importance of added vitamin B_{12} . The vaccine-testing laboratory of the US Department of Agriculture maintains its hamster-virulent challenge strains by continuous passage through hamsters of homogenised liver tissue (Nervig, 1975, personal communication). This might explain why cell replication of *canicola in vitro* is so drastically impaired by a lack of vitamin B_{12} . Bovine albumin can be obtained in a state of purity (Ellinghausen, 1968) sufficient for the demonstration of the vitamin B_{12} requirement of leptospire, but although the thiamine in P-80 medium can be shown to be required for maximal growth, the amount of this vitamin in various batches of bovine albumin sometimes makes it almost impossible to demonstrate an absolute requirement. If leptospire can be grown in a medium containing only albumin, vitamin B_{12} and thiamine and found to be fully immunogenic, the importance of albumin will be even more conclusively proved.

We need to understand what constitutes the endogenous nutrient stores of leptospire and how such stores are related to survival and possibly virulence.

The hardy nature of *canicola* was seen by its survival in diluted kidney homogenate, unsupplemented simplified medium and vitamin B₁₂-deleted medium. Growth in organ culture might be worthy of study. Miller, Miller and White (1966) have shown that growth of leptospires occurs in the presence of mammalian cells produced by tissue culture.

Further work is needed on growth of leptospires on solid agar, particularly with strains that are known to fluctuate in virulence in the laboratory. The uniformity of leptospiral colonial morphology offers no prospect of assistance in differentiating virulent from avirulent progeny. When strain *canicola* (NADL A-13) was studied, the overwhelming proportion of the cell population appeared highly virulent. Similar studies with leptospires that produce kidney infection but not death might be rewarding. Studies will also be required with different media to see if specific nutrients encourage a more rapid death rate of genetically virulent organisms than of genetically avirulent organisms.

How long cells can remain virulent without progressive growth is probably a function of endogenous stores. Ellinghausen (1973a) has described the maintenance of virulence of a strain of serotype *pomona* during prolonged storage in BSAD. Refined studies of such cells are needed to see if their chemical composition would shed some light on the problem. The preservative activity of BSAD may be related to some of the micronutrients reported in bovine serum albumin by Hanson and Ballard (1968) and Ellinghausen (1968).

Wilson and Foster (1971) have described limited proteolysis of bovine albumin by an enzyme present in commercial albumin preparations. This should be taken into account in view of the growth of leptospires that can be achieved in media from which ammonium chloride and polysorbate 80 have been omitted.

As Pearce and Lowrie (1972) have stated, we need a better understanding of the factors that control bacterial multiplication *in vivo*, and those that control host specificity. Simultaneous studies of host and infective agent are thus required.

SUMMARY

Leptospira interrogans serotype *canicola* (strain NADL A-13) grew from inocula as small as two cells in liquid polysorbate 80 medium (P-80 medium), in P-60, P-40 and P-20 media, and in P-80 medium from which polysorbate, NH₄Cl or thiamine had been omitted. It grew well initially in vitamin B₁₂-deleted P-80 medium, but only with inocula as large as 26×10^4 cells per ml.

P-80 medium lacking both polysorbate and NH₄Cl supported light growth from small inocula, but the omission of thiamine and vitamin B₁₂ in addition seriously affected the properties of the medium. Where readily detectable growth did not develop in liquid nutrient-deleted medium, viable organisms could often be demonstrated indirectly by subculture to semisolid medium, and their occurrence was influenced by the presence of albumin, thiamine, and vitamin B₁₂.

Growth on semisolid media was comparable with that in liquid media

of similar composition. The absence of polysorbate 80, thiamine, or vitamin B₁₂ prevented the appearance of Dinger's zones of growth from small inocula.

Antigenic composition as measured by microscopic agglutination tests with homologous and heterologous antisera was not appreciably affected by repeated subculturing in various complete and incomplete media.

Homogenates of infected-hamster-kidney tissue in bovine serum-albumin diluent still contained viable organisms after 60 days' storage at 23–25°C. Organisms derived from this material after 3 and 16 days' storage showed no loss of virulence. Organisms grown in artificial culture showed no loss of virulence after storage in bovine albumin diluent or phosphate buffer for 7 days at 23–25°C.

Cultures of the organism survived without loss of virulence for 15 months in 13 semisolid media of differing complexity. Single colonies derived from five different solid media were grown in semisolid forms of the parent media and stored at 23–25°C for 10 months without loss of virulence.

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