Deoxyribonucleic Acid Hybridization Among Selected Leptospiral Serotypes

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The deoxyribonucleic acids (DNA) from phenotypically unusual *Leptospira* strains were compared to each other and to reference strains of existing genetic groups. In the "pathogenic" genetic complex, three groups emerged as genetically distinct. Representative examples of these groups were *bataviae* strain Van Tienen, *javanica* strain Veldrat Bataviae, and *ranarum* strain Iowa City Frog. The serologically different *muenchen* strain Muenchen 90C, *icterohaemorrhagiae* strain RGA, and *kabura* strain Kabura were found to be closely related to *bataviae* strain Van Tienen. The "saprophytic" genetic complex also contained three inter-related groups. The representative examples were *patoc* strain Patoc I, *codice* strain CDC, and Turtle strain A-183. Strain 3055 of serotype *illini* appeared to have unique nucleotide sequences and was placed in a third genetic complex of its own. Partial relatedness between DNA could be emphasized by increasing the salt concentrations in the incubation media. This could not be attributed to the methods of annealing but appeared to be dependent upon the genetic relatedness between the heterologous DNA.

The genus *Leptospira* can be separated into two major divisions, the pathogenic and the so-called "saprophytic" or "biflexa" leptospires. The latter are usually found in fresh surface waters and are rarely found in animals. In addition to infectivity, various phenotypic properties serve to separate members of the two divisions (18). Using deoxyribonucleic acid (DNA) base composition determinations and specific DNA-DNA annealing tests in agar matrices, Haapala et al. (7) demonstrated genetic differences between, as well as within, serotypes in the two divisions. Two distinct genetic groups were demonstrated among selected strains within each of the pathogenic and biflexa serotypes. The serological relationships among leptospires did not necessarily denote genetic relatedness; however, strains of the same or very similar serotypes appeared to be in the same genetic group. The two genetic groups of pathogenic leptospires could be differentiated from each other and from the two biflexa groups by a number of phenotypic characteristics which also served as a basis for the leptospiral groups described by Johnson and Harris (9). The principal differentiating attributes were lipase production and relative resistance to the bacteriostatic action of 8-azaguanine (AZA) and 2,6-diaminopurine (DAP).

The genetic groups of pathogenic strains represented by strains of serotypes *bataviae* and *javanica* corresponded to the Johnson and Harris biological groups 1 and 2, respectively. Group 1 strains were sensitive to AZA and DAP and had lipase activity, whereas group 2 strains were sensitive to AZA, resistant to DAP, and lacked lipase activity. The two genetic groups of biflexa strains were phenotypically indistinguishable and fit into the Johnson and Harris biological group 3, which was characterized by resistance to both purine analogues and by positive lipase activity.

The purpose of this report is to decipher genetic interrelationships of representative strains within the four genetic groups and among additional phenotypically distinct strains of leptospires. (This work was presented in part at the International Symposium on Leptospiroses, Bratislava-Smolenice, Czechoslovakia, 11-13 September 1973.)

MATERIALS AND METHODS

Bacterial strains. Van Tienen (serotype *bataviae*) and Veldrat Bataviae 46 (serotype *javanica*) are representative strains of the genetic groups of pathogenic leptospires; Patoc I (serotype *patoc*) and the CDC strain of serotype *codice* (11) are representative

of the two genetic groups of biflexa strains (9). Pathogenic leptospiral strains Kabura (serotype *kabura*) and Muenchen C90 (serotype *muenchen*) were selected because they did not fit into the biological groups described by Johnson, and Harris (9). Strain Kabura was sensitive to both AZA and DAP and lacked lipase activity (9). Strain Muenchen C90 was resistant to DAP, sensitive to AZA, and had lipase activity (R. Johnson, personal communication).

Two amphibian isolates, Iowa City Frog (serotype *ranarum*) and Turtle A-183 (serotype undetermined), were included because of their unique host origin and possession of attributes characteristic of both pathogenic and saprophytic leptospires. The Iowa City Frog strain was originally isolated from pooled kidneys of frogs (4,5). It behaved like a pathogen in its relative susceptibility to AZA, CuSO_4 , and NaHCO_3 (2) and in its serological affinity with a pathogenic leptospira strain, 267-1348, isolated in Malaysia (unpublished data); however, the strain was serologically distinct and was classified as a new serotype, *ranarum*, by Babudieri (1). The frog strain resembled biflexa strains in its ability to multiply at low temperatures (5) and its inability to establish infection in laboratory animals (1,4). Strain Turtle A-183 was isolated from the cloaca of a turtle in an enzootic area of leptospirosis in Illinois (R. D. Andrews, Ph.D. Thesis, University of Illinois, Urbana, 1966). Its guanine plus cytosine content, 36.0%, was suggestive of pathogenic leptospires on the basis of previous work (7), but it had cultural and biochemical attributes of biflexa strains.

Strain 3055, isolated in 1965 from the urine of a bull in Illinois (Lyle Hanson, unpublished data), is unique in its ability to grow in ordinary Trypticase soy broth without the addition of serum or serum components (H. C. Ellinghausen, personal communication); it is also the only leptospira to have a phage associated with it (12). Like biflexa strains, its growth is uninhibited by relatively high concentrations of purine analogues and divalent copper ions. It also did not cause illness in calves or laboratory rodents (15). Strain 3055 was serologically distinct when tested against an array of 55 diverse serotype antisera from all known pathogenic serogroups. It was also unrelated to selected serologically distinct strains of biflexa. It has recently been identified as a new serotype and was designated serotype *illini* (16).

Strain RGA was included because of its historical importance as the oldest extant representative strain of *L. interrogans*, serotype *icterohaemorrhagiae*. *Proteus mirabilis*, an organism with a DNA nucleotide base ratio similar to that of leptospiral DNA but which is completely unrelated to the genus *Leptospira*, was used as the source of control DNA.

Cultural conditions. The identity of each culture was confirmed serologically by agglutination tests with specific antisera. All cultures were grown at 30 C in modified Ellinghausen and McCullough albumin fatty acid broth (17). Cultures were harvested by centrifugation during the late logarithmic growth and examined for contaminating microorganisms by the use of appropriate cultural techniques and microscope examinations. Radioactive DNA was prepared by the addition of 0.25 mCi of $[8\text{-}^3\text{H}]\text{adenine}$ (54 to 58

mCi/mmol, Schwarz Mann, Orangeburg, N. J.) or 2.5 mCi of $[^3\text{H}]\text{adenine}$ (G) (6.0 Ci/mmol, New England Nuclear, Boston, Mass.) per liter of culture media prior to bacterial inoculation. After purification, the DNA were sheared and denatured by the method of Rogul et al. (13) and are henceforth designated as DNA*. The specific activities (counts per minute per microgram) of the DNA containing $[^3\text{H}]\text{adenine}$ were as follows: *bataviae*, 10,325; *javanica*, 8,522; *patoc*, 8,162; and *codice*, 18,681. The specific activities of the DNA containing $[8\text{-}^3\text{H}]\text{adenine}$ were: *ranarum*, 52,992; A-183, 30,576; and *illini*, 19,985.

DNA extraction. The DNA extraction method of Marmur (10) was followed through the ribonuclease step. Subsequent purification followed the method of Rogul et al. (13). The purified DNA preparations were dissolved in 0.1 standard saline citrate (SSC = 0.15 M NaCl, 0.015 M sodium citrate) and refrigerated until used.

Determination of base composition. The buoyant densities of the nucleic acids were determined by equilibrium density gradient centrifugation in CsCl solutions using a Spinco model E analytical ultracentrifuge. The buoyant densities were converted to percent nucleotide base composition expressed as percent guanine plus cytosine (G + C) by the method of Schildkraut et al. (14).

DNA binding on membrane filters. Annealing tests were performed by the method of Denhardt (3). The nucleic acids were denatured by heating to 100 C in a $0.1 \times \text{SSC}$ solution for 10 min and quenching in an ice bath. Thirty micrograms of DNA was placed on 25-mm, 0.45- μm pore size membrane filters (MPF: Millipore Corp., Bedford, Mass.) by aspirating 5 ml of $6 \times \text{SSC}$ containing the DNA through the filter. After drying the filters, annealing tests were carried out using the preincubation media of Denhardt containing $3 \times \text{SSC}$ or $6 \times \text{SSC}$. DNA* was added in a ratio of 1:30 to the amount of filter-immobilized DNA. After 16 h of incubation at 65 C, the filters were washed with $3 \times$ or $6 \times \text{SSC}$ and dried. From experiments performed with homologous and nonrelated DNA preparations, 65 C was determined to be the optimum temperature for specific binding. The amount of radioactivity retained on each MPF was determined by scintillation counting. The scintillating fluid was a toluene solution containing 4 g of Omnifluor per liter (New England Nuclear, Boston, Mass.). Relatedness was calculated from the following equation: $[(\text{counts/min retained on MPF in the heterologous reaction}) / (\text{counts/min retained on MPF in the homologous reaction})] \times 100 = \text{percentage of genetic relatedness}$.

DNA binding in agar. Quantitative DNA duplex formation in agar matrices was determined using a procedure similar to that of Rogul et al. (13). Approximately 0.3 g of agar containing 90 μg of DNA were incubated with 3 μg of DNA*. The DNA in $2 \times$, $3 \times$, or $6 \times \text{SSC}$ was annealed in small screw-capped vials for 16 h at 65 C. The contents of the vials were then transferred to thermally controlled, water-jacketed columns. Solutions of $2 \times$, $3 \times$, or $6 \times \text{SSC}$ were used to wash unbound DNA* from the agar at 65 C; the columns were then allowed to cool, and 2-ml washes of $0.1 \times \text{SSC}$ were used to elute the bound

DNA* as the temperature of the columns was raised from 50 to 75 C. The eluants were collected in scintillation vials, and the radioactivity was determined by scintillation counting in a toluene-Triton X-100 (2:1 vol/vol) solution containing 4 g of Omnifluor per liter.

Relatedness was calculated from the following equation: [(total counts/min of bound DNA* eluted from the agar in the heterologous reaction)/(total counts/min of bound DNA* eluted from the agar in the homologous reaction)] $\times$ 100 = percentage of genetic relatedness.

RESULTS AND DISCUSSION

DNA duplex formation on MPF. The annealing tests in a previous study (7) were done in

an agar matrix prepared with double-strength standard saline citrate solution (2 $\times$ SSC). For the present studies, the Denhardt method (3) was first selected because it had considerable advantages over the agar matrix method in time and material requirements. In Denhardt's original publication, the final salt concentrations in the incubation media were difficult to determine because of the various methods of DNA denaturation employed. The final concentrations appeared to range from 3 to 6 $\times$ SSC. Generally, 6 $\times$ SSC is considered desirable to retard the leaching of immobilized DNA from the filters (2, 6, 8).

The results of annealing tests in 3 $\times$ and 6 $\times$ SSC are summarized in Table 1. When complementary base sequences were compared,

TABLE 1. Comparative homology of leptospiral DNA in 3 $\times$ and 6 $\times$ SSC incubation solvents^a

Complex	Serotype immobilized DNA	Percent homology of reference DNA and incubation solvent							
		<i>bataviae</i> *		<i>javanica</i> *		<i>patoc</i> *		<i>codice</i> *	
		3 $\times$ SSC	6 $\times$ SSC	3 $\times$ SSC	6 $\times$ SSC	3 $\times$ SSC	6 $\times$ SSC	3 $\times$ SSC	6 $\times$ SSC
"Pathogens"	<i>bataviae</i>	100	100	35.2	50.8	10.2	4.8	8.4	8.0
	<i>muenchen</i>	99.0	97.5	34.7	62.6	8.9	4.7	12.2	6.2
	<i>icterohaemorrhagiae</i>	92.6	80.0	29.9	51.4	9.4	4.1	5.1	6.6
	<i>kabura</i>	71.0	75.5	28.3	54.6	9.1	4.9	7.3	8.1
	<i>javanica</i>	41.7	48.8	100	100	8.9	4.0	6.6	5.3
	<i>ranarum</i>	31.9	45.3	39.8	55.3	10.6	4.1	6.3	6.6
"Saprophytes"	<i>patoc</i>	7.3	4.8	3.0	5.7	100	100	35.7	66.9
	<i>codice</i>	4.6	8.7	3.1	6.7	29.0	42.7	100	100
	Turtle (A-183)	8.7	8.0	5.7	5.8	35.6	32.8	59.1	54.4
New	<i>illini</i>	2.8	1.6	4.9	1.4	2.6	1.7	1.7	1.7
Control (<i>Proteus mirabilis</i>)		3.6	2.9	1.8	1.6	4.6	1.9	3.3	2.4

^a Annealing performed on MPF.

TABLE 2. Comparative homology of leptospiral DNA by different methods

Complex	Serotype immobilized DNA	Percent homology of reference DNA and incubation matrix					
		<i>bataviae</i> *			<i>patoc</i> *		
		Agar 2 $\times$ SSC ^a	MPF 3 $\times$ SSC	MPF 6 $\times$ SSC	Agar 2 $\times$ SSC ^a	MPF 3 $\times$ SSC	MPF 6 $\times$ SSC
"Pathogens"	<i>bataviae</i>	100	100	100	0	10.2	4.8
	<i>javanica</i>	13	41.7	48.8	6	8.9	4.0
"Saprophytes"	<i>patoc</i>		7.3	4.8	100	100	100
	<i>codice</i>		4.6	8.7	19.0	29.0	42.7

^a Data from Haapala et al. (7).

TABLE 3. Characteristics of leptospiral DNA duplex formation in agar matrix under variable concentrations of SSC incubation media

Serotype radioactive DNA	Serotype immobilized DNA	2 × SSC			3 × SSC			6 × SSC		
		Bind-ing (%)	E _m ^b (C)	Homol-ogy (%)	Bind-ing (%)	E _m ^b (C)	Homol-ogy (%)	Bind-ing (%)	E _m ^b (C)	Homol-ogy (%)
<i>bataviae</i> *	<i>bataviae</i>	42.2	65.8	100	44.9	65.5	100	52.9	65.7	100
	<i>icterohaemorhagiae</i>		63.2	92.4		62.6	102		62.2	98.7
	<i>javanica</i>		55.1	30.7		54.5	41.0		54.6	50.2
<i>ranarum</i> *	<i>ranarum</i>	52.8	67.0	100						
	<i>bataviae</i>		53.7	62.6						
	<i>javanica</i>		54.4	40.9						
<i>patoc</i> *	<i>patoc</i>	46.6	68.1	100	57.0	67.7	100	61.9	68.5	100
	<i>codice</i>		56.7	20.5		55.9	24.5		55.2	31.2
	A-183 ^a		57.5	15.9		56.3	26.8		56.6	28.4
Turtle A-183*	A-183	24.9	67.0	100						
	<i>patoc</i>		57.0	40.3						
	<i>codice</i>		59.4	50.1						
<i>illini</i> *	<i>illini</i>	23.3	68.7	100						

^a The serotype of this strain has not been determined.^b E_m, Thermal elution median.

there were sharply delineated groups with 70% or greater relative homology within complexes of obviously related organisms but less than 13% relative binding between strains of these different complexes. With little exception, increasing the salt concentration of the solution in which annealing took place appeared to induce greater binding among DNA of partial homology. This was especially striking when we compared work previously performed in 2 × SSC in agar columns (Table 2; reference 7). Thus, the radioactive *bataviae* DNA fragments had apparent comparative homologies of 13% in 2 × SSC, 41.7% in 3 × SSC, and 48.8% in 6 × SSC with the immobilized *javanica* DNA. Similarly, the *patoc* DNA* appeared to have homologies of 19% in 2 × SSC, 29% in 3 × SSC, and 42.7% in 6 × SSC with the immobilized *codice* DNA. Increase in DNA binding under increased salt concentration did not occur between DNA from organisms which were definitely not genetically related (e.g., the *bataviae* DNA* and *Proteus mirabilis* DNA, Table 1).

This increased retention of DNA* raised two important questions: (i) did the salt actually increase DNA annealing, or (ii) was there a salt effect on the MPF which indirectly affected DNA retention on the filters? To solve these

questions, the three salt concentrations were used to anneal DNA in agar matrices (Table 3). The results were then compared to those obtained on membrane filters (Table 1) and were similar.

Figure 1 shows the affect of temperature and salt concentration during the annealing process on the subsequent elution of DNA* from the homologous *bataviae* DNA. The elution profiles illustrated a trend whereby, regardless of salt concentration, as the temperature of incubation increased, the total amounts of duplexed DNA increased up to a point (65 C incubation) where two specific elution peaks were observed. At higher incubation temperatures, annealing conditions were more restrictive, resulting in less binding and a series of erratic peaks at the higher elution temperatures. Nonetheless, the elution profiles were indicative of a group of DNA within the species which preferentially bound at high salt concentrations and eluted at low temperatures. Whether this was preferential binding of DNA which was mismatched, damaged, or a unique segment is not known. In our tests, the incubation temperature for optimal annealing appeared to be 65 C. When other serotypes or unrelated species were compared to the *bataviae* DNA (Fig. 2), nonspecific aggregation of DNA was appropriately ruled

FIG. 1. Comparison of elution profiles of bataviae DNA* with agar-embedded homologous DNA in incubation fluids of different concentrations. Annealing temperatures were (A) 60 C, (B) 65 C, and (C) 70 C. (D) Characteristics derived from the elution profiles A, B, and C. Elution medians and total counts per minute bound at different salt concentrations.

FIG. 2. Comparison of elution profiles of bataviae DNA* incubated at 65 C with agar-embedded heterologous DNA in incubation fluids of varying salt concentrations. (A) bataviae DNA* tested against javanica DNA, (B) bataviae DNA* tested against codice CDC DNA, (C) bataviae DNA* tested against *P. mirabilis* DNA, (D) characteristics derived from the elution profiles A, B, and C. Elution medians, percent homology, and total counts per minute bound at different salt concentrations.

out (Fig. 2C and D). The high salt concentrations obviously encouraged greater annealing of partially related DNA sequences (Fig. 2 A, B, and D) but not between the *bataviae* DNA and obviously unrelated sources (Fig. 2C and D).

These effects were also studied on duplexes formed by the *bataviae* DNA* and a genetically closely-related strain, *icterohaemorrhagiae* RGA.

The elution profile of the *bataviae* DNA* annealed to the DNA of the RGA strain is very similar to the homologous reaction of the *bataviae* DNA (Fig. 1B). If anything, the initially eluted peak (Fig. 3A) was more pronounced. When the reaction was repeated and elution was examined at lower wash temperatures (Fig. 3B), there was an obvious elution of DNA* at the lower temperatures but not the distinct peak which occurred when the washing began at 50 C.

In contrast, when the homologous DNA from serotype *patoc* was annealed at 65 C (this was the compromise temperature for organisms having DNA of 36 to 39% G + C), the amount of DNA* which eluted at low temperatures from a homologous duplex was negligible (Fig. 4A). Low-temperature elution of the *patoc*

DNA* apparently only occurred with duplexes formed with genetically heterologous but distinctly related strains.

The heterologous reaction of the *patoc* DNA*/*codice* DNA (Fig. 4B) was further investigated at very low elution temperatures (Fig. 4C). It was plain to see that small amounts of the *patoc* DNA* which annealed had clung to the *codice* DNA with great tenacity over a wide temperature range.

A number of strains which showed partial genetic homology were examined at varied salt concentrations in the agar columns. There was no doubt that the total bound DNA* increased with increasing salt concentration, but the thermal elution median (E_m) remained relatively stable in the homologous reaction. As might be expected, the E_m drops just slightly in the heterologous reactions as the salt concentration increases.

When all the results were assembled and compared, it was apparent that the Denhardt technique, as well as the agar-column method, provided a means of distinguishing genetically distinct strains or, under modified salt conditions, of emphasizing certain partial homologies. From Table 1 the almost negligible DNA

FIG. 3. Elution profile of *bataviae* DNA* incubated at 65 C with *icterohaemorrhagiae* RGA DNA embedded in agar. Incubation fluids were varied as shown in figures. (A) Elution profile between 50 C and 75 C. (B) Elution profile between 34 C and 60 C.

FIG. 4. Elution profiles of patoc DNA* incubated at 65 C with homologous and heterologous agar-embedded DNA under varying salt concentrations. (A) patoc DNA* tested against patoc DNA, (B) patoc DNA* tested against codice CDC DNA. Elution profile between 50 C and 76 C. (C) patoc DNA* tested against codice CDC DNA. Elution profile between 30 C and 75 C. (D) Characteristics derived from the elution profiles A and B. The elution medians, percent homology, and total counts per minute bound at different salt concentrations.

annealing of the pathogenic representatives (*bataviae* and *javanica*) with the saprophytic strains of either *patoc* or *codice* reflects the great genetic gulf between strains of the two major complexes, whereas Table 3 presents data which emphasizes the partial homologies of groups within the complexes. These data were summarized in Table 4.

Strain 3055 (serotype *illini*) appeared to have distinct nucleotide sequences and may represent a new "complex." Its DNA had little complementarity with DNA* from strains of *bataviae*, *javanica*, *ranarum*, *patoc*, *codice*, or A-183. The DNA annealing patterns of the *icterohaemorrhagiae* strain were indistinguishable from that of the serotype *bataviae* strain. This was confirmed in thermal-elution profiles using the *bataviae* DNA* and bound *icterohaemorrhagiae* RGA DNA (Fig. 3). Similarly, the *muenchen* strain appeared to have the same nucleotide sequence as the reference *bataviae* strain. These findings negated the usefulness of DAP as a differentiating character for genetic groups (9).

Among other pathogenic strains, partial homologies were demonstrated by immobilized DNA from strains of *ranarum* and *kabura* against the reference strains of *bataviae* and *javanica* DNA*. Strain Turtle A-183 had partial genetic homology with the two representative strains of the *biflexa* genetic group (Tables 1 and 3).

The genetic interrelatedness of the *illini* and *ranarum* strains and Turtle A-183, with each other and with the representative strains of the two pathogenic and two saprophytic genetic groups (Table 1), was reexamined by annealing DNA in agar columns (2 × SSC incubation fluid). As mentioned before, this method provided generally acceptable stringent conditions for duplex formation, and the derived elution profiles gave a more definitive account of the relationships among the population of sheared DNA duplexes. These results were also listed in Table 3. The Turtle A-183 and Frog (*ranarum*) strains were found to be genetically distinct entities within their complex. The findings indicated the presence of 7 distinct

TABLE 4. Percent relative homology of leptospiral DNA of representative serotypes

Complex	Serotype radioactive DNA ^a	Percent homology with immobilized DNA from serotype						
		<i>bataviae</i>	<i>javanica</i>	<i>ranarum</i>	<i>patoc</i>	<i>codice</i>	A-183	<i>illini</i>
"Pathogens"	<i>bataviae</i> * ^b	100	41.7	31.9	7.3	4.6	8.7	2.8
	<i>javanica</i> * ^b	35.2	100	39.8	3.0	3.1	5.7	4.9
	<i>ranarum</i> * ^c	62.6	40.9	100	2.6	1.2	1.6	0.8
"Saprophytes"	<i>patoc</i> * ^b	10.2	8.9	10.6	100	29.0	35.6	2.6
	<i>codice</i> * ^b	8.4	6.6	6.3	35.7	100	59.1	1.7
	Turtle A-183* ^c	2.7	7.9	6.2	40.3	50.1	100	5.4
New	<i>illini</i> * ^c	2.9	6.6	3.3	6.4	9.0	6.4	100

^a Nonrelated DNA binding with *Proteus mirabilis* ranged from 0.5 to 3.4% in agar matrix and from 1.8 to 3.6% on membrane filters.

^b DNA annealed on membrane filter in 3 × SSC solvent.

^c DNA annealed on agar matrix in 2 × SSC solvent.

TABLE 5. Phenotypic characteristics of genetically-distinct serotypes^a

Characteristic	Pathogenic complex			Saprophytic complex			<i>illini</i> ^b
	<i>bataviae</i>	<i>javanica</i>	<i>ranarum</i>	<i>patoc</i>	<i>codice</i>	A-183	<i>illini</i>
"Pathogenic"	+	+	?	—	—	?	?
Resistance to AZA	S	S	S	R	R	R	R
Resistance to Cu ions	S	S	S	R	R	R	R
Resistance to NaHCO ₃	S	S	S	R	R	R	
Growth at 13 C	—	—	+	+	+		
Lipase	+	—		+	+	+	
% G + C	35.3	39.9	41.2	38.3	38.0	36.0	53.0 ^c

^a Abbreviations: R, resistant; S, sensitive; AZA, 8-azaguanine.

^b Ritchie et al. (12) demonstrated a phage in strain 3055 of serotype *illini*.

^c Apparent base composition derived from buoyant-density data.

genetic groups of leptospires which could be assembled into 3 major "complexes" on the basis of DNA relatedness. Between complexes, strains have little or no demonstrable genetic relatedness. Two of the three complexes could each be segregated into groups of strains which have partial DNA homologies. Complexes of pathogenic and saprophytic leptospires were each comprised of 3 genetic groups. A third complex comprised the strain of serotype *illini*. The relatedness of the *kabura* strain to other pathogenic genetic types was not determined. Table 4 was designed to bring together the information in both Tables 1 and 3, whereas the phenotypic characteristics in Table 5 were assembled because of the data derived from these DNA tests. The DNA nucleotide base composition of *illini* strain 3055 was particularly striking and may reflect the presence of a phage genome (12) or some other condition. This strain requires a DNA examination by other definitive methods. We also concluded that the percent G + C of DNA could not be correlated with pathogenicity.

It is interesting that 3 or 4 strains which were selected for further genetic studies because of their unusual origin or unusual phenotypic attributes proved to be genetically distinct. The characters which prompted their selection may in combination suffice to distinguish strains between, but not within, "complexes."

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