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Miscellanea.

Source Determination of Blood Meal in Sandflies (Phlebotominae) in Yugoslavia (Dobricky County).

2,760 haemoprecipitin tests.

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(Received December 27th, 1950.)

Cases of Kala-azar in Yugoslavia have been observed in earlier times, e.g., before World War I, but lately this disease has assumed a form of minor epidemics. In 1948 there were recorded and under treatment at Nish Children's Hospital (Dobricky county) 42 cases of Kala-azar and in 1949 another 17. The age of patients ranged from 3 months to 13 years. A systematic examination made by a field team in Dobricky county (middle Serbia, population about 6,000) revealed 39 suspected cases out of 999 children examined. Of these children 17 had to be sent to Nish Children's Hospital and there the diagnosis of Kala-azar was confirmed. We believe that, had we examined a greater number of children and over a wider area, more cases would have been found.

As regards the transmitting of Kala-azar, it is well known that many insects were thought to be the means of it, until, through the works of *Acton*, *Sinton* and *Shortt*, the evidence pointed strongly to *Phlebotomus* species. However, even this *Phlebotomus* theory was disputed by experimental work of *A. G. Brooks* (1950) who states boldly that the *Phlebotomus* theory has received a "knock down and knock out".

In a study of diseases transmitted by Phlebotominae, mere registration of locality in which Phlebotominae are found, of their abundance and seasonal distribution do not supply sufficient data. More important factors are the species which are met with in those regions. In Kala-azar epidemics in Yugoslavia we gathered evidence which is conflicting. There are regions where Phlebotominae fauna is abundant, yet no Kala-azar cases or very few indeed were found and vice versa. Thus, although many Phlebotominae species can be infected experimentally and in nature with *Leishmanias*, only suitable species of Phlebotominae may start an epidemic. Through the works and in the opinion of *Simić* the following species come into consideration as Kala-azar vectors in Yugoslavia. For the province of Serbia *P. chinensis* var. *simici* Nitzulescu and *P. perfiliewi* Parrot; for Macedonia *P. chinensis* var. *simici*, *P. major* Annandale and *P. perfiliewi*; for Montenegro mainly *P. major* and for the Dalmatian coast *P. major* and *P. perniciosus* var. *tobby*.

In an attempt to answer the question of the epidemiology of Kala-azar as well as to establish an epidemiological link between *Phlebotomus* species, source of blood meal ingested and distribution of Kala-azar, we have systematically examined 460 Phlebotominae. Capturing was done early in the morning inside human dwellings as well as in such places as stables, cellars, latrines, livestock sheds, churches, etc. Only well-filled females were chosen and they were dissected the same day. The oozing blood was collected on a slip of filter paper. All dissections had to be carried out carefully, taking precautions not to damage genital parts which along with pharynx are of importance in deter-

mining the species variety. The filter paper slips imbibed with blood were marked and stored in test tubes for precipitin test examination in laboratory.

In determining the source of blood meal, all haemoprecipitin tests were performed by ring method and our previous experience with *Anopheles* work in Macedonia (1937) proved helpful. The other precipitin test methods were tried also (Gradwohl, icebox, etc.), but in our hands the ring method proved superior. Special precipitin test tubes (3×50 mm.) were used. The capillary pipette method, although a ring method and described as rough and ready, may be useful, we think, with *Anopheles* work, but we did not find it suitable for Phlebotominae work. The readings are in our opinion more difficult and there are many indefinite and doubtful results.

The specific antisera which we have produced in our laboratories were: antihuman (titer 1-12,000), antiox (1 : 10,000), antihorse (1 : 10,000), antipig (1 : 14,000), antidog (1 : 16,000) and antisheep (1 : 18,000).

In preparing our antisera we have greatly profited by experimental experiences of *H. Proom* (B. Wellcome labs., 1943) as well as of other authors. The rabbits were injected intramuscularly with the alum precipitated serum of the animal whose antiserum we wish to obtain. The dosage was 2-3 injections of 10 c.c. serum with an interval of 10-14 days after each injection.

The technique used in performing precipitin test was as follows: All blood dilutions were made by soaking filter paper slips in 5 c.c. of distilled water, whereby we obtained a dilution of 1/500 to 1/1000; reckoning that every *Phlebotomus* gave us approximately 0.005 to 0.01 c.c. of blood. Because there was some opalescence, all blood dilutions were filtered until a perfectly clear solution was obtained. Now 0.5 c.c. of clear dilution was poured into each of 6 small test tubes (3×50 mm.). Into the 7th and 8th test tubes, 0.5 c.c. of distilled water was poured and these served as controls. Into the first 6 test tubes, 0.1 c.c. of specific antiserum was carefully poured respectively for human, horse, pig, dog, ox and sheep blood. Into the 7th test tube (antiserum control) we chose to put 0.1 c.c. of antihuman serum and into the 8th test tube 0.1 c.c. of blood dilution (antigen control). All reactions were performed at room temperature (18-20° C.) and read off by naked eye in reflected light. Readings were done within few minutes up to one hour. After this period all subsequent reactions were disregarded and the control test tubes remained clear. Pipettings were done by capillary pipettes using always separate pipettes for each antiserum. This procedure is rather different from the capillary method with *Anopheles* blood work, where the same pipettes are used irrespectively of blood dilution and after each serial test simply shaken off and wiped with a piece of cloth.

The results we have obtained are shown in tables I, II and III. The *Phlebotomus* species we have found (*Simić, Živković*) are as follows: Out of 460 female *Phlebotomi*, 271 belonged to *P. papatasii* Scopoli; 144 to *P. perfiliewi* Parrot; 28 to *P. chinensis* var. *simici* Nitzulescu and 11 to *P. major* Annandale. The remaining 6 phlebotomae were ill marked or badly damaged and had to be discarded.

As regards to locality where they were caught, we present the following table.

From this table we see that the predominant species within human dwellings is *P. papatasii*, though—but in a smaller number—other species may also be found. The species of Phlebotominae found within human dwellings in order of frequency are as follows: *P. papatasii*, *P. perfiliewi*, *P. chinensis* and *P. major*.

Species	Within human dwellings		Outside human dwellings	
	Total number	%	Total number	%
<i>P. papatasii</i>	193	83.9	78	34.7
<i>P. perfiliewi</i>	25	10.8	119	53.1
<i>P. chinensis</i>	8	3.5	20	8.9
<i>P. major</i>	4	1.9	7	3.1

P. papatasii, as is well known, is a dominant species with a strong inclination for human habitations, whilst other species seem to prefer microclimatic conditions outside of these.

The source of blood meal revealed in Phlebotominae points to a preference for human blood, regardless of localities where caught. This holds good even when the blood meal was made on two or more animals.

Entering into analysis of results (table I) we perceive that

a) *P. papatasii* caught within human dwellings most frequently ingested human blood and then of other animals. Those caught outside human habitations have also in a large percentage (42.4%) partaken of human blood. Rather high percentages are shown for ox blood (20.5%), horse blood (14.1%) and rarest for dog blood (5.1%).

b) *P. perfiliewi* shows preference for human blood and takes a high percentage, regardless whether they were caught within houses or outside. The rarest findings were for horse and sheep blood.

c) *P. chinensis* var. *simici* analogously to other species shows also a tendency for human blood irrespective of locality where caught, proportion being of 50% in houses to 38% outside these. In our findings we see exceptionally high percentages for dog blood (25% and 10%), whilst lowest for ox and sheep blood.

d) *P. major*, although represented by a small number, is also a human blood sucker. Next to human blood it chooses ox and horse blood but never dog blood.

In 28 out of 460 Phlebotominae examined (6.08%), in spite of careful and controlled work, we could not trace their source of blood meal. Thus, we obtained no reaction with the six antisera we used and the control test tubes remained clear. We suppose it might have been the blood of birds, reptiles or small rodents, whose antisera we did not have at our disposal. A direct microscopic examination would probably have revealed nucleated erythrocytes but these, on account of rapid digestion within the Phlebotomus, would have to be done in the field and on the same day when the Phlebotominae are caught. However, we were not equipped for this.

Curiously enough 23 Phlebotominae (about 5%) partook of their blood meal from 2 animals and in one instance from 3 different animals.

Close relation between human and animal blood ingested by Phlebotominae creates a possibility for transmission of many blood diseases and Kala-azar likewise. In *P. chinensis* var. *simici* and *P. perfiliewi* we frequently met with dog blood which points to them as the commonest species of Phlebotominae playing an important role as vector of *Leishmania Donovanii* in this country.

TABLE I.
Phlebotominae caught within human habitations.
Source of blood meal.

Species	Human		Horse		Dog		Ox		Sheep		Pig		Unknown		Re- mark:
	Total number	%	Total number	%	Total number	%	Total number	%	Total number	%	Total number	%	Total number	%	
<i>P. papatasii</i>	121	63.2	15	7.8	4	2.1	23	11.8	7	3.6	8	4.1	11	5.6	Non specific reactions, missing <i>Phlebotominae</i> and other pitfalls -3-
<i>P. perfiliewi</i>	19	76	0	0	3	12	1	4	0	0	1	4	1	4	
<i>P. chinensis</i> var. <i>simici</i>	4	50	0	0	2	25	0	0	1	12	1	12	0	0	
<i>P. major</i>	3	75	1	25	0	0	0	0	0	0	0	0	0	0	

TABLE II.
Phlebotominae caught out of human habitations.
(Stables, sheds, cellars, barns, churches, etc.)
Source of blood meal.

Species	Human		Horse		Dog		Ox		Sheep		Pig		Unknown		Re- mark:
	Total number	%	Total number	%	Total number	%	Total number	%							
<i>P. papatasii</i>	33	42.4	11	14.1	4	5.1	16	20.5	5	6.4	5	6.4	3	3.8	Non specific reactions, missing <i>Phlebotominae</i> and other pitfalls -6-
<i>P. perfiliewi</i>	55	46.2	6	5.1	18	15.1	17	14.8	3	2.3	10	8.4	10	8.4	
<i>P. chinensis</i> var. <i>simici</i>	7	35	3	15	2	10	5	25	1	5	0	0	2	10	
<i>P. major</i>	3	42.8	2	28.5	0	0	2	28.5	0	0	0	0	0	0	

TABLE III.
Phlebotominae where source of blood meal shows to be of two or more animals.

Species	Human dog		Human sheep		Human pig		Human ox		Human horse		Horse pig		Horse ox		Human horse pig	
	within houses	out	w.h.	out	w.h.	out	w.h.	out	w.h.	out	w.h.	out	w.h.	out	w.h.	out
<i>P. papatasii</i>	3	1	0	3	2	0	2	0	1	0	0	0	0	0	0	1
<i>P. perfiliewi</i>	2	2	0	0	1	0	1	0	0	0	1	0	0	0	0	0
<i>P. chinensis</i> var. <i>simici</i>	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
<i>P. major</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0

We are of the opinion that greater evidence would be obtained if further work was conducted on similar lines including a greater number of Phlebotominae in regions where numerous cases of Kala-azar exist.

Summary.

Epidemiology of Kala-azar was studied and attempts were made to establish an epidemiological link between Phlebotomus species, sources of their blood meal and distribution of Kala-azar. Investigations were made in 11 villages (with a total population of about 6,000) of Dobricky county. 460 female sandflies, caught early in the morning after their blood meal, were examined. Species of Phlebotominae found were: *P. papatasii* Scopoli, *P. major* Annandale, *P. chinensis* var. *simici* Nitzulescu, *P. perfiliewi* Parrot. The most abundant were *P. papatasii* and *P. perfiliewi*, the rarest *P. major* (only 12).

P. papatasii and *P. perfiliewi* take mostly human blood, the other species mostly animal blood (dog, horse, cattle, pig, sheep). Blood in 24 out of 460 sandflies was not identified and might be that of birds, reptiles and small rodents.

Particular attention was given to the preparation of specific precipitin antisera. Sandflies, as known, contain small quantities of blood and for exact work high titer antisera are required. These sera must be strictly specific inasmuch as the animals in question are closely related (horse-mule, sheep-goat, cow-buffalo).

Method for preparing antisera: Rabbits were injected intramuscularly with alum precipitated serum. Potent and highly specific antisera were thus obtained, with a titer reaching up to 18,000. Ring method with small test tubes (3 × 50 mm.) was best for precipitin tests.

Our experiments are considered preliminary and of local application. Investigations covering a wider range and including a greater number of sandflies would give more data and thus more satisfactory results.

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