A GROUP OF MICROORGANISMS TRANSMITTED HEREDITARILY IN TICKS AND APPARENTLY UNASSOCIATED WITH DISEASE.*

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PLATES 33 TO 35.

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In the course of a study of the relation of *Rickettsia ruminantium*[1] to heartwater, it was found that a microorganism previously reported in *Amblyomma hebræum*[2] was present not only in the eggs of adult females, but also in larvæ hatched therefrom and in nymphæ. With the unusual opportunities afforded for the investigation of all stages in the life history of ticks in the laboratory of the Department of Agriculture of the Union of South Africa, an attempt was made to ascertain whether this microorganism in *Amblyomma hebræum* afforded an isolated example of hereditary transmission or was one of a group of parasites thus transmitted.

Attention was first directed toward the study of unfed larvæ because the probability seemed to be a strong one that any microorganisms found in them must have been inherited through the eggs, since the larvæ had hatched out from eggs deposited by the female in sterile test-tubes plugged with cotton which were carefully kept from contamination.

Soon somewhat similar microorganisms characterized by relatively

* Third contribution by the South African Expedition of The Rockefeller Institute for Medical Research.

The experiments were made in the Laboratory of the Department of Agriculture at Onderstepoort. Cordial thanks are due to the Government of the Union of South Africa, to Sir Arnold Theiler, and to the members of his staff for the many courtesies extended.

[1] Cowdry, E. V., *J. Exp. Med.*, 1925, xlii (in press).

[2] Cowdry, E. V., *J. Exp. Med.*, 1923, xxxvii, 431.

large size as compared with most *Rickettsiæ*, great pleomorphism, Gram-negative properties, and intracellular habitat were observed in both smears and sections of the unfed larvæ of *Boophilus decoloratus*, *Hæmophysalis leachi*, *Rhipicephalus appendiculatus*, *Rhipicephalus evertsi*, and *Rhipicephalus simus*, in addition to *Amblyomma hebræum*. Fortunately these six species of ticks were being reared in the laboratory for other purposes so that material was at hand in which to trace the history of the microorganisms through some of the subsequent stages of development. Photographs of the microorganisms, as seen in smears, are given in Figs. 1 to 6; camera lucida drawings, illustrating their degree of pleomorphism, are reproduced in Figs. 7 to 12; and their appearance in sections is shown by the drawings in Figs. 13 to 18.

The survey was extended by the inclusion of certain phases in the life history of other ticks available in the laboratory and by a re-examination of specimens previously described.[1,2] In this way it was found that microorganisms of the same general type, and probably also transmitted hereditarily, were present in at least sixteen species. These are denoted by the plus (+) signs in Table I, which gives full information regarding the material examined. Unless there is a statement to the contrary, it is to be understood that the ticks were obtained in South Africa. To these the laboratory numbers are appended for reference. The methods employed in handling the larvæ, nymphæ, and adults, and the technique applied were the same as those used in an earlier study on *Rickettsia ruminantium* in ticks.[1]

The microorganisms could not be detected by direct illumination of living cells with or without the addition of vital dyes with sufficient facility and constancy to delimit their properties, although in favorable preparations they were clearly recognizable.

Dark-field examination[3] of living cells revealed only a few of the morphological forms in the case of each species of tick. In some cases the entire microorganisms appeared brightly luminous, while in others the luminous material was confined to a thin marginal layer. In no instance was definite motility observed.

In air-dried smears after fixation for about 15 minutes in absolute alcohol the microorganisms stained light red or pink with Giemsa's stain, that is to say colored much more faintly than most bacteria, and they did not possess the sharp

[3] With the precautions previously described.[1]

contours often seen in the case of bacteria. They were very pleomorphic, varying from spherules to straight and curved rods and filaments. They were found to be larger than most *Rickettsiæ* particularly in their diameter, which varied from about 0.3μ to 1.5μ. Filaments were frequently met with, sometimes as much as 5μ in length, or longer, in the case of *Boophilus decoloratus*, in which branching was noted (Fig. 2).

Group Characteristics of the Microorganisms in Unfed Larvæ.

Through the examination of living cells, of smears, and of sections it was found that the microorganisms in different species resembled each other in (1) their relatively large size as compared with most *Rickettsiæ;* (2) the restriction of their intracellular habitat to the Malpighian tubules, and to the eggs of the females; and (3) certain of their staining reactions.

Their ends were generally uniformly and evenly rounded; but occasionally they were sharply pointed. This slight affinity for stains and pleomorphism served as a basis of differentiation between the microorganisms and certain refractile coccoid, rod-like, and dumb-bell-shaped concretions contained in the Malpighian tubules which otherwise might have constituted a source of error.

After fixation in Regaud's fluid the microorganisms in sections were colored dark red or purple by Giemsa's stain. They were uniformly Gram-negative in both smears and sections. They stained feebly by Unna's alkaline methylene blue and other basic aniline dyes. They were colored faintly red by Pappenheim's pyronine-methyl green method. By all methods of staining, when they were embedded in cytoplasm, some were observed to exhibit distinct halos. It was also in sections that their intracellular habitat was most clearly distinguished. Their location was very characteristic, being restricted to the cytoplasm of the epithelial cells of the Malpighian tubules of larvæ, nymphæ, and adults. Occasionally they were seen in the cells lining the rectal sac, which is a continuation of the Malpighian tubules. They were found also in the eggs of adults of ten species. In no instance were they found to invade other tissues.

Differential Properties of the Microorganisms in the Different Species of Unfed Larvæ.

While possessing certain group characteristics in common, as indicated above, it was found possible to distinguish clearly the microorganisms present in each species of tick by characteristic differences in their morphology alone.

TABLE I.

Material Examined.

Species.	Unfed larvæ.	Nymphæ.	Adults.
Amblyomma americana +			Sections of 2 from Columbia, S. C., 5 from Washington, N. C., and 10 from Baton Rouge, La., fixed in Regaud's fluid.[2]
Amblyomma hebræum +	Smears of 25 and sections of 25 fixed in Regaud's fluid (517); dark-field examination; also 112 engorged larvæ prepared by a variety of methods.[1]	300 prepared by many methods, some infected with heartwater.[1]	Sections of 20 fixed in formalin and Regaud's fluid;[2] also sections of 6 infected with heartwater.[1]
Amblyomma maculatum —			Sections of 6 from Vancleave, Miss., fixed in formalin and Regaud's fluid.[2]
Amblyomma tuberculatum —			Sections of 1 from Perkinston, Miss., fixed in formalin and Regaud's fluid.[2]
Argas mineatus —			Sections of 2 from Southerland, Fla., fixed in Zenker's and Regaud's fluids.[2]
" *persicus* +	Smears of 25 and sections of 25 fixed in Regaud's fluid; also dark-field examination.		

Boophilus decoloratus +	Smears of 50, sections of 50, and dark-field examination of several, some infected with redwater (517, 570, 608, 622).		Sections of 10 fixed in formalin and Regaud's fluid.[2]
Dermacentor albipictus −			Sections of 2 from Regina, Canada, fixed in formalin and Regaud's fluid.[2]
Dermacentor variabilis +			Sections of 5 from Urbana, Ill., and 8 from College Park, Md., fixed in formalin and Regaud's fluid.[2]
Dermacentor venustus +			Sections of 9 from Hamilton, Mont., and 2 from Wawawai, Wash., fixed in Regaud's fluid and some infected with Rocky Mountain spotted fever.
Hæmophysalis leachi +	Smears of 25 and sections of 25 fixed in Regaud's fluid; also dark-field examination (629).		
Hæmophysalis leporis palustris −			Sections of 19 from Raleigh, N. C., fixed in formalin and Regaud's fluid.[2]
Hyalomma ægyptium +	Sections of 25 fixed in alcohol.		Sections of 5 fixed in Regaud's fluid; also dark-field examination.

TABLE I—*Concluded.*

Species.	Unfed larvæ.	Nymphæ.	Adults.
*Margaropus annulatus** +			Sections of 8 from Columbia, S. C., 10 from Washington, N. C., and 2 from Baton Rouge, La., fixed in Regaud's fluid.[2]
Margaropus annulatus australis +			Sections of 6 from Jamaica, fixed in formalin and in Regaud's fluid. Sections of 3 from Trinidad, fixed in the same way.[2]
Margaropus winthemi −	Sections of 25 fixed in alcohol.		
Ornithodorus megnini +	Sections of 25 fixed in Regaud's fluid; also dark-field examination.		
Ornithodorus moubata −	Sections of 25 fixed in alcohol.		
Ornithodorus turicata +			Sections of 1 from Arizona, fixed in Regaud's fluid.[2]
Rhipicephalus appendiculatus +	Smears of 25 and sections of 25 fixed in Regaud's fluid; also dark-field examination (621).		Smears of 1 and sections of 5 fixed in Regaud's fluid. Some infected (?) with anaplasmosis, others exhibited many bacteria in tracheal system (602, 612).

Rhipicephalus capensis −	Sections of 25 fixed in alcohol.		
Rhipicephalus evertsi +	Smears of 10, also dark-field examination; sections of 25 fixed in alcohol and of 25 fixed in Zenker's fluid (633).	Smears of 2 and sections of 11 fixed in Regaud's fluid and infected with East Coast fever (644, 645, 646, 650).	Sections of 20 fixed in formalin.[2]
Rhipicephalus pulchellus +		Sections of 2 fixed in Regaud's fluid and infected with East Coast fever (618).	Smears of 1, infected with East Coast fever (639).
Rhipicephalus sanguineus +			Sections of 14 from Hawaiian Islands fixed in formalin and Regaud's fluid;[2] also smears of 3 (555).
Rhipicephalus simus +	Smears of 25 (624), sections of 25 fixed in alcohol, and of 25 fixed in Regaud's fluid; also dark-field examination.	Smears of 2 and sections of 2 fixed in Regaud's fluid and infected with anaplasmosis (611).	Smears of 7 and sections of 6 fixed in Regaud's fluid and infected with anaplasmosis (561, 564).

* The generic term *Margaropus* is retained intentionally. It was supplied by Dr. F. C. Bishopp of the Bureau of Entomology of the U. S. Department of Agriculture and was employed by me in an earlier paper.[2]

Thus, in *Amblyomma hebræum* (Figs. 1, 7, and 15) the microorganisms were noticeably plumper and of larger diameter than in the case of any of the other ticks. They were, moreover, often grouped together in an unique fashion suggestive of multiplication by some kind of budding.

In *Boophilus decoloratus* the microorganisms differed from those in the other ticks by showing marked red- and blue-staining materials (Fig. 8), by the fact that some of the morphologic types were colored rather intensely by Giemsa's stain (Fig. 8), and by their branching (Fig. 2). They were never grouped together after the fashion of the microorganisms in *Amblyomma hebræum*, *Hæmophysalis leachi*, *Rhipicephalus appendiculatus*, and *Rhipicephalus evertsi*.

The microorganisms in *Hæmophysalis leachi* were easily distinguished from all the rest by a peculiar tendency of the filaments to be grouped side by side, by the presence of unusually marked chromophobe areas within the filaments, and by the occurrence of forms indicative of the possibility of multiplication by longitudinal splitting (Figs. 3, 9, and 18).

In *Rhipicephalus appendiculatus* the microorganisms were of particularly small size and were grouped distinctively (Figs. 4, 10, and 14). In *Rhipicephalus evertsi* still another type of grouping was manifest (Figs. 5, 11, and 16), which called to mind certain forms of *Rickettsia lectularia* as described by Hertig and Wolbach[4] and represented in their Figs. 25 and 26, while *Rhipicephalus simus* differed sharply from the two above mentioned species of the same genus by the fact that its contained microorganisms were not grouped in any way other than in diplo formation (Figs. 6, 12, and 17). These microorganisms in *Rhipicephalus simus* more closely resembled those in *Boophilus decoloratus* than those in any of the other five species to which reference has already been made; but they differed from the microorganisms in *Boophilus decoloratus* by their failure to exhibit red- and blue-staining materials and in the absence of branching forms.

On the basis of these observations it would be quite possible to identify unfed larvæ of any of these species by an examination of the contained microorganisms.

In addition, microorganisms having somewhat similar general properties were observed in unfed larvæ of *Argas persicus* and *Ornithodorus megnini*, but differing slightly as between their respective hosts. The failure to detect like microorganisms in alcohol-fixed specimens of *Hyalomma ægyptium*, *Margaropus winthemi*, *Ornithodorus moubata*, and *Rhipicephalus capensis*, as noted in Table I, may have been due to imperfect preservation and consequently cannot be interpreted as conclusive evidence of the absence of microorganisms of this general type in the unfed larvæ of these species.

Evidence of the Hereditary Transmission of the Microorganisms.

That the finding of the microorganisms in the unfed larvæ of eight

[4] Hertig, M., and Wolbach, S. B., *J. Med. Research*, 1924, xliv, 329.

species,[5] in the eggs of ten species,[6] and in smears of the adults of four others,[7] is indicative of hereditary transmission on a fairly large scale is supported by tracing them through the entire life cycle of three species.

Thus, in *Amblyomma hebræum*, *Rhipicephalus evertsi*, and *Rhipicephalus simus* they were observed not only in the Malpighian tubules of unfed larvæ, carefully protected from bacterial contamination, but also in the Malpighian tubules of nymphæ and in the Malpighian tubules and eggs of adult females. The series of preparations of *Amblyomma hebræum* made at close intervals for the study of *Rickettsia ruminantium* was particularly valuable. It was observed that throughout the periods of engorgement and moulting the microorganisms exhibited with constancy the morphological and microchemical properties already alluded to, so that the likelihood of error in their identification was almost negligible.

Occurrence of the Microorganisms.

Although technical difficulties prevented the detection of the microorganisms in all the individual ticks examined, favorable preparations always revealed their presence in 100 per cent of the ticks when prolonged search was made, and it is believed that they were invariably present in the specimens of the following species which were studied: *Amblyomma americana*, *Argas persicus*, *Boophilus decoloratus*, *Dermacentor variabilis*, *Amblyomma hebræum*, *Dermacentor venustus*, *Hæmophysalis leachi*, *Hyalomma ægyptium*, *Margaropus annulatus*, *Margaropus annulatus australis*, *Ornithodorus megnini*, *Ornithodorus turicata*, *Rhipicephalus appendiculatus*, *Rhipicephalus evertsi*, *Rhipicephalus sanguineus*, and *Rhipicephalus simus*.

The failure to observe them, as indicated by the negative signs (−) in Table I in *Amblyomma maculatum*, *Amblyomma tuberculatum*, *Argas mineatus*, *Dermacentor albipictus*, *Hæmophysalis leporis palustris*, *Margaropus winthemi*, *Ornithodorus*,

[5] *Amblyomma hebræum*, *Argas persicus*, *Boophilus decoloratus*, *Hæmophysalis leachi*, *Ornithodorus megnini*, *Rhipicephalus appendiculatus*, *Rhipicephalus evertsi*, and *Rhipicephalus simus*.

[6] *Amblyomma americana*, *Amblyomma hebræum*, *Boophilus decoloratus*, *Dermacentor variabilis*, *Dermacentor venustus*, *Margaropus annulatus*, *Margaropus annulatus australis*, *Ornithodorus turicata*, *Rhipicephalus evertsi*, and *Rhipicephalus sanguineus*.

[7] *Argas persicus*, *Rhipicephalus appendiculatus*, *Rhipicephalus pulchellus*, and *Rhipicephalus simus*.

moubata, and *Rhipicephalus capensis* is open to two interpretations, either that they are absent, or that further search in better preparations would bring them to light. In favor of the latter hypothesis may be mentioned the fact that the material available for their study was much less satisfactory than that of the species in which the microorganisms were actually discovered, and, further, the consideration that these ticks all belong to genera other species of which were found to contain the microorganisms.

There is no indication that the season of the year has any influence upon the association of the microoganisms with their arachnid hosts.

TABLE II.

Geographic Distribution of the Microorganisms.

Similar micro-organisms.	In.	Collected at.
Present.	*Amblyomma americana.*	1. Columbia, S. C., by Dr. W. K. Lewis. 2. Washington, N. C., by Dr. Hartwell Robbins. 3. Baton Rouge, La., by Dr. E. Pegram Flower.
"	*Dermacentor variabilis.*	1. Urbana, Ill., by Dr. W. P. Flint. 2. College Park, Md., by Dr. E. N. Cory.
"	*Dermacentor venustus.*	1. Hamilton, Mont., by Dr. R. R. Parker. 2. Wawawai, Wash., by Dr. A. L. Melander.
"	*Margaropus annulatus.*	1. Columbia, S. C., by Dr. W. K. Lewis. 2. Washington, N. C., by Dr. Hartwell Robbins. 3. Baton Rouge, La., by Dr. E. Pegram Flower.
"	*Margaropus annulatus australis.*	1. Jamaica, British West Indies, by Dr. B. E. Washburn. 2. Trinidad, Leeward Islands, by Mr. John L. Rice.
"	*Rhipicephalus sanguineus.*	1. Honolulu, Hawaiian Islands, by Mr. N. H. Cowdry. 2. Onderstepoort, Union of South Africa.

The evidence at hand hardly permits a statement of the geographical distribution of the microorganisms. In other words, it is possible that South African ticks are especially prone to harbor microorganisms of this kind by reason of favorable climatic conditions and other unknown factors and that the same species elsewhere might not possess them. Against this interpretation, however, was the observation of apparently identical microorganisms in individuals of a single

species collected in widely different localities. For convenience, the available information is summarized in Table II.

It will be noted that the majority of the ticks examined came from tropical or temperate regions, but this does not necessarily mean that temperature is in any sense a controlling factor in the distribution of the microorganisms, since they also occur in specimens of *Dermacentor venustus* originating at Hamilton, Montana, where the winter temperature falls far below zero.

From the geographic point of view, Honolulu and Onderstepoort are very widely separated. Not only is their location almost antipodal, but Honolulu is hot and humid and situated at sea-level, while Onderstepoort is at an altitude of about 4,000 feet, somewhat cooler, and very dry in winter, when the specimens were collected. Yet examples of *Rhipicephalus sanguineus* collected at these two places harbor microorganisms which are indistinguishable in their morphology and tinctorial properties, in their location in the tissues, and in the probability of their hereditary transmission.

DISCUSSION.

The exact systematic position of these hereditarily transmitted microorganisms is not easily established, but the possibility of the existence of a relationship to the *Rickettsiæ* and to certain symbionts may be mentioned.

The Gram-negative properties, intracellular habitat, and bacterium-like shape of the microorganisms and their presence in arthropods would perhaps justify their provisional inclusion under the general heading of *Rickettsiæ*.[3] It will be recalled that this characterization of *Rickettsia* was used as a working basis by Hertig and Wolbach[4] in their studies on *Rickettsia*-like microorganisms in insects and that these studies led them to suggest the propriety of still further restricting the term to proved pathogenic microorganisms having the following properties: small size, pleomorphism, slight affinity for aniline dyes, and intracellular habitat. Since the microorganisms under discussion in this paper are of relatively large size, sometimes 0.5μ or more in diameter, and are apparently non-pathogenic, they obviously do not measure up to the requirements of this more exacting definition of *Rickettsia*.

The microorganisms resemble the symbionts of certain blood-feeding flies and lice in size and to some extent in staining reactions but differ from them sharply in so far that they occur in the Malpighian tubules instead of the alimentary tract and by their failure to give rise to the peculiar organ-like structures called mycetomes.

It is difficult to compare them with the symbionts of ticks because the latter have only been very inadequately described in the literature. Thus, Godoy and Pinto[8] in 1922 mentioned the presence of a microorganism in the ovaries of certain species of Brazilian Ixodidæ. They named it "*Ixodisymbionte*" and noted that it was first discovered by R. Koch in *Rhipicephalus*. Unfortunately Godoy and Pinto gave but few criteria for the identification of the microoganisms and their paper was not illustrated. A careful search for an earlier and more complete description by Koch was unsuccessful. The only other reference to symbionts in ticks which I have been able to find was published in the same year by Buchner[9] and consists merely of a few lines in a paper devoted to a general discussion of symbiosis. It is a record of the occurrence of a symbiont within the egg cells and the cells of the Malpighian tubules of an unspecified species of the genus *Ixodes* and is accompanied by two figures.

Although it cannot be said definitely, it does appear probable that the microorganisms described in this paper belong to the same general category as those previously reported by Godoy and Pinto and by Buchner. If the former are correct in their reference to Koch it would seem that this investigator deserves credit not only for his well known discovery of the symbionts of the tsetse flies but also for the first description of symbionts in ticks which we are now able to recognize as very widely distributed in both the Argasidæ and the Ixodidæ.

SUMMARY.

1. Pleomorphic, bacterium-like, Gram-negative, intracellular microorganisms, which stained much less intensely with ordinary dyes than

[8] Godoy, A., and Pinto, C., *Brazil-med.*, 1922, ii, 335; reviewed in *Trop. Dis. Bull.*, 1923, xx, 474.

[9] Buchner, P., *Naturwissenschaften*, 1922, x, 1.

most bacteria were found in sixteen species of ticks comprising examples of the Argasidæ and the Ixodidæ.

2. In six of these species studied intensively slight differences in the microorganisms were detected, sufficient to permit identification of the vectors by microscopic examination of the microorganism alone.

3. No evidence was seen of injury to the tissues of the arachnid hosts of the microorganisms other than that incident to mechanical distention of the cells containing them.

4. The detection of the microorganisms in the eggs of ten species, in the unfed larvæ of eight species, and at nearly related stages throughout the life cycle of three others leads to the conclusion that they are transmitted hereditarily.

EXPLANATION OF PLATES.

PLATE 33.

Photomicrographs of the microorganisms, made with apochromatic objective 3 mm., 1.40 aperture, and compensating ocular 8, giving a magnification of 1,400 diameters, of air-dried smears of unfed larvæ, fixed in absolute alcohol and colored by Giemsa's stain.

FIG. 1. *Amblyomma hebræum.*

FIG. 2. *Boophilus decoloratus.*

FIG. 3. *Hæmophysalis leachi.*

FIG. 4. *Rhipicephalus appendiculatus.*

FIG. 5. *Rhipicephalus evertsi.*

FIG. 6. *Rhipicephalus simus.*

PLATE 34.

Drawings of selected microorganisms from smears of unfed larval ticks to show extent of their pleomorphism. All the drawings were made from air-dried smears, fixed in alcohol and colored by Giemsa's stain with apochromatic objective 1.5 mm., compensating ocular 8, and camera lucida, and have been slightly reduced in reproduction.

FIG. 7. *Amblyomma hebræum.*

FIG. 8. *Boophilus decoloratus.*

FIG. 9. *Hæmophysalis leachi.*

FIG. 10. *Rhipicephalus appendiculatus.*

FIG. 11. *Rhipicephalus evertsi.*

FIG. 12. *Rhipicephalus simus.*

PLATE 35.

All the figures are drawings of portions of the Malpighian tubules of unfed larval ticks made with apochromatic objective 1.5 mm., compensating ocular 8, and camera lucida and have been slightly reduced in reproduction.

FIG. 13. *Boophilus decoloratus.*

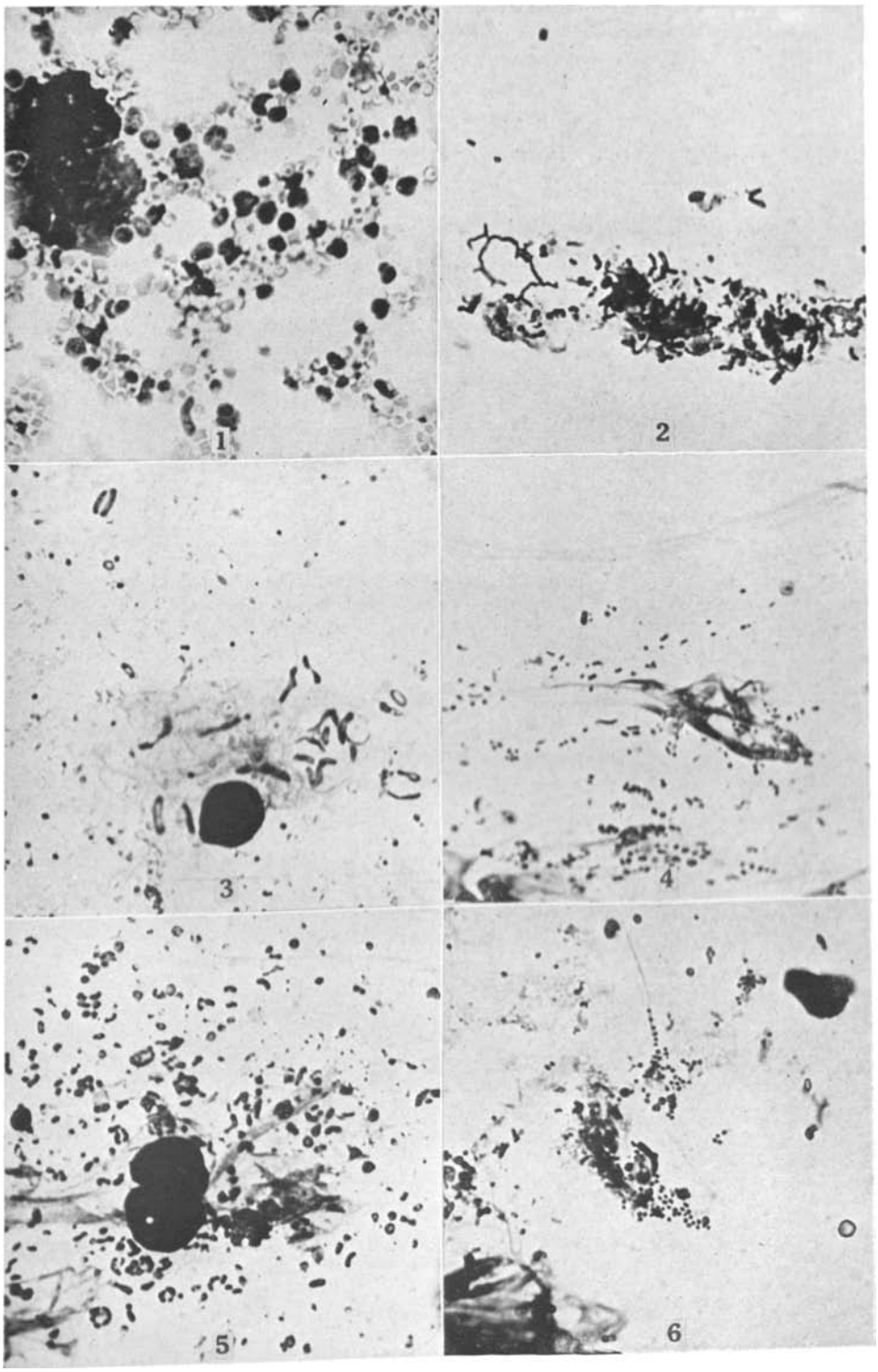
FIG. 14. *Rhipicephalus appendiculatus.*

FIG. 15. *Amblyomma hebræum.*

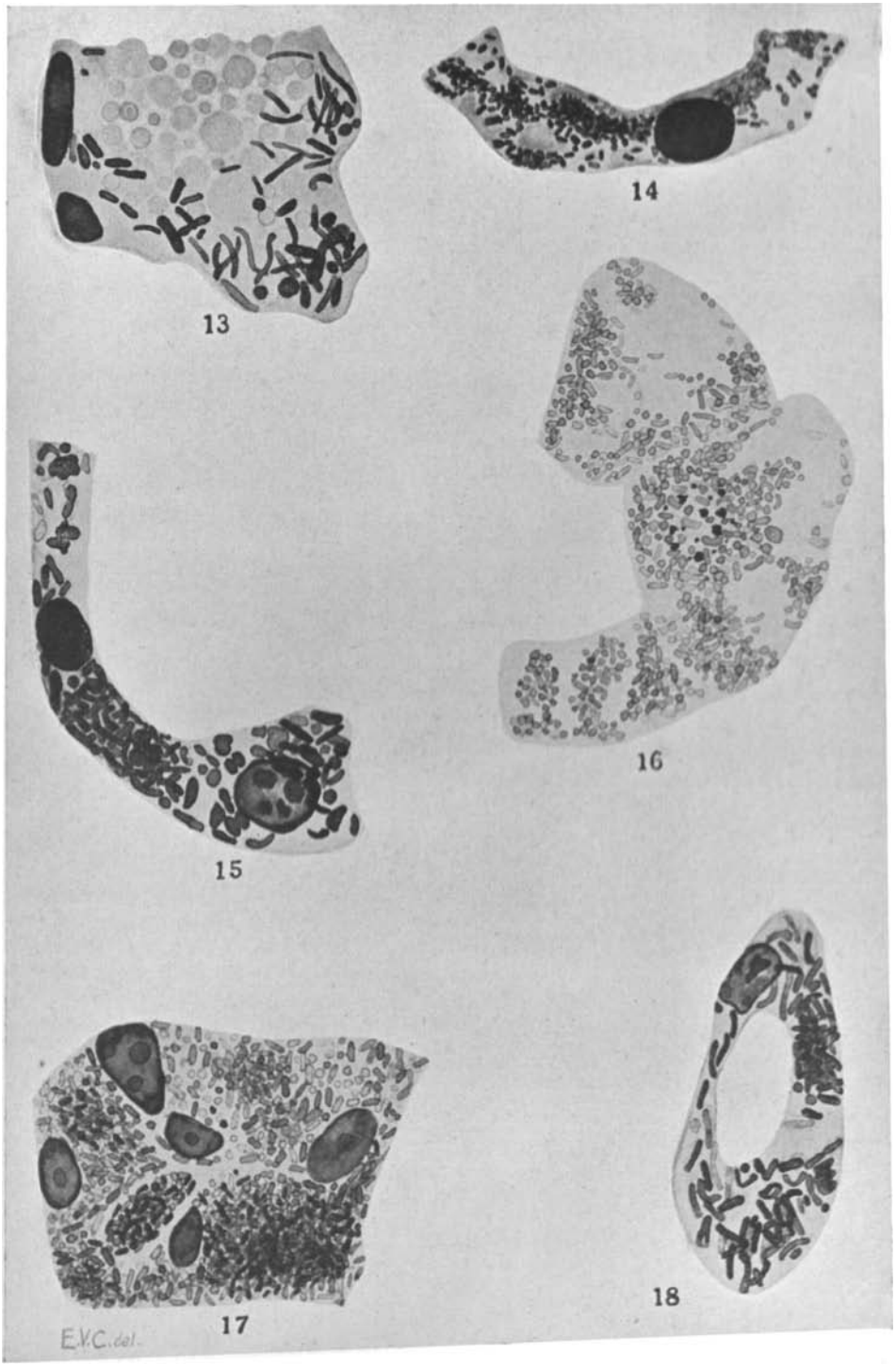
FIG. 16. *Rhipicephalus evertsi.*

FIG. 17. *Rhipicephalus simus.*

FIG. 18. *Hæmophysalis leachi.*



(Cowdry: Microorganisms in ticks.)



(Cowdry: Microorganisms in ticks.)