Bull Org. mond. Santé
Bull. Wld Hlth Org. } 1966, **35**, 155-164

In Vitro Cultivation of the Rickettsial Agent of Trench Fever *

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Although trench fever appears to be endemic in many areas of the world, recognition of the disease has been handicapped by the difficulties of making a clinical diagnosis and the unavailability of a simple laboratory procedure to establish the etiology. The author describes a method for the in vitro *cultivation of* Rickettsia quintana *that provides a relatively simple means for the laboratory diagnosis of trench fever.* R. quintana *can be propagated with ease from the blood of patients directly on blood agar incubated at 37°C for 12-14 days under a gas tension of 5 % CO_2 in air. The number of rickettsiae circulating in the patient's peripheral blood can be quantitated. The protracted rickettsiaemia in trench fever makes for a relatively long period during which blood culture can be usefully employed.*

In the course of studies with the method described, it was found that erythrocytes contain a factor (or, possibly, factors) essential for multiplication of R. quintana *; this factor is cryostable and thermostable and may be haemoglobin. Blood serum also promotes multiplication.*

Although *Rickettsia quintana* would appear to exist in ecological equilibrium with its human host and louse vector in many areas of the world, recognition of trench fever has been mainly confined to the large-scale epidemics of the First and Second World Wars. Between epidemics the disease subsides into an endemic state and reports of primary cases disappear from the medical literature. The difficulty of making a clinical diagnosis of trench fever in the absence of an epidemic and the unavailability of a simple laboratory procedure to establish the etiology of the infection significantly contribute to the lack of recognition of clinical disease in endemic areas during interepidemic periods.

In recent studies, *R. quintana* was propagated directly on cell-free media from the blood of trench fever patients (Vinson & Fuller, 1961; Vinson et al.[2]), and proof that the micro-organisms so cultivated were identical to *R. quintana* was obtained by the fulfilment of Koch's postulates in human volunteer subjects (Vinson, 1964; Vinson et al.[2]). From these observations have evolved procedures for the laboratory diagnosis of trench fever and for studies of its epidemiology.

The medium originally described for the *in vitro* propagation of *R. quintana* consisted of Difco blood-agar base [3] enriched with 10% fresh defibrinated horse or human blood. Incubation was at 35°C in a moist atmosphere of 5% CO_2 in air. While the medium satisfactorily supported growth of a European strain of *R. quintana*, it did not allow of subcultivation of Mexican strains. A preliminary analysis indicated that fresh blood contained a substance, presumably complement, inhibitory of the Mexican strains. Substitution of 6% horse serum inactivated at 56°C for 30 minutes and 4% washed horse erythrocytes eliminated the inhibitory effect and permitted luxuriant growth of both European and Mexican strains. Use of haemolysed erythrocytes produced a translucent medium facilitating visualization of the microscopic colonies of *R. quintana*, and incubation at 37°C accelerated the rate of multiplication. Rabbit blood could be substituted

* Revised version of a paper presented at the First International Congress of Parasitology, held in Rome, Italy, in September 1964. This study was conducted under the auspices of the Commission on Rickettsial Diseases of the Armed Forces Epidemiological Board and was supported in part by the Office of the Surgeon General, US Department of the Army.

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[2] Vinson, J. W., Varela, G. & Molina, C.—in preparation.

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for horse or human blood. *R. quintana* did not multiply without added CO_2 nor under anaerobic conditions achieved by replacing air with nitrogen.

These observations find application for the laboratory diagnosis of trench fever. The cultural procedures provide a relatively simple technique for isolation of *R. quintana in vitro* from the blood of trench fever patients and for the propagation of rickettsiae in quantities sufficient to prepare antigens for serological detection of the disease. Serological studies will be reported separately. The present paper describes methods for propagating *R. quintana in vitro* and presents data on factors in the medium essential for multiplication of *R. quintana*.

MATERIALS AND METHODS

Media

The basic medium consisted of Difco blood-agar base (beef-heart infusion, tryptose, NaCl, and agar); 6% horse serum inactivated at 56°C for 30 minutes; and 4% horse erythrocytes washed 3 times with phosphate-buffered saline (pH 7.2) and haemolysed either with distilled water or by rapid freezing to −60°C and rapid thawing at 37°C. The fresh defibrinated horse blood routinely used yielded serum contaminated with haemolysed erythrocytes. In testing components of the media, therefore, non-haemolysed serum was obtained from clotted horse blood.

The volume and ionic concentration of the experimental media were kept, where possible, comparable to those of the basic medium by the addition of various solutions known by previous studies not to be injurious to *R. quintana*. In determining the optimal concentration of erythrocytes, the volumes were kept constant where required by the addition of salt K7G (Bovarnick & Miller, 1950), having the following composition: KCl, 0.118 M; NaCl, 0.0072 M; Na_2HPO_4, 0.0076 M; KH_2PO_4, 0.0040 M; potassium glutamate, 0.0049 M; pH 7.0. In determining the effect of serum in the medium, the serum was replaced by salt K7G, by distilled water, or by a solution composed of sucrose, 0.218 M; KH_2PO_4, 0.00376 M; K_2HPO_4, 0.0071 M; potassium glutamate, 0.0049 M; pH 7.0 (Bovarnick, Miller & Snyder, 1950) hereinafter referred to as sucrose-PG. To determine the cryostability of the essential factor or factors in erythrocytes, the erythrocytes were (*a*) rapidly frozen in a dry-ice/alcohol bath to −60°C and rapidly thawed at 37°C, or (*b*) frozen slowly to −30°C and thawed at room temperature. To determine the thermostability of this factor (or factors), the erythrocytes were held at 56°C or at 100°C (boiling water-bath) for 30 minutes, or were autoclaved at 15 lbf/in² (1.05 kgf/cm²) for 15 minutes. To learn whether haemoglobin could replace erythrocytes in the medium, 4 mg haemoglobin/ml were tested in media containing serum concentrations ranging from 0 to 33%. In a related experiment the haemoglobin varied from 0 mg/ml to 10 mg/ml while the serum concentration was constant at 20%. The twice recrystallized human haemoglobin [1] was dissolved in distilled water to a concentration of 20 mg/ml, Seitz-filtered, and added in appropriate volumes to the liquid agar media at 45°C immediately before pouring the plates.

Incubation conditions

The standard incubation was at 37°C for 5-7 days in a moist atmosphere of 5% CO_2 in air. Petri-dish cultures were placed in desiccator jars containing saline, and air was replaced by 5% CO_2 in air from a tank containing the compressed mixture of gases. *R. quintana* used to prepare stock suspensions were grown in rubber-stoppered milk-dilution bottles in which 5% CO_2 in air had replaced air.

Strains of R. quintana

In all experiments but one the Fuller strain of *R. quintana* was used (Vinson & Fuller, 1961). In a single experiment the Guadalupe strain was employed, isolated directly on basic medium from the blood of a trench fever patient in Mexico City and passaged on this medium (Vinson et al.[2]). The stock pools of the rickettsiae used in the experiments were prepared from micro-organisms propagated on basic medium under the standard incubation conditions described above. Colonies were scraped into sucrose-PG, and the rickettsiae were mechanically dispersed in an Omnimix [3] and washed by two cycles of centrifugation for 30 minutes at 30 160 *g* in the cold. Aliquots of the washed, concentrated rickettsiae, suspended in sucrose-PG, were shell-frozen in a dry-ice/alcohol bath and stored at −60°C.

Titration of R. quintana *and test methods*

The approximate numbers of viable rickettsiae in the stock suspensions were estimated by titration on

[1] Nutritional Biochemicals Corp., Cleveland, Ohio, USA.

[2] Vinson, J. W., Varela, G. & Molina, C.—in preparation.

[3] Manufactured by Ivan Sorvall, Inc., Norwalk, Conn., USA.

basic medium in 60 mm × 15 mm plastic Petri dishes. Log dilutions of the stock suspension (considered to be 10^0) were prepared in sucrose-PG, and 0.05-ml volumes of each dilution were inoculated on to each of three plates. Following standard incubation, colonies on plates showing between one and approximately 400 colonies were counted. Counts were accomplished with a simple stereoscopic microscope at 20× magnification, using both transmitted and reflected light, by simultaneously obliterating each colony with a platinum needle and registering it on a hand tally. Results are expressed as the mean number of colonies on the three plates inoculated with each dilution of the stock suspension.

To test the effect of various substances on the growth of *R. quintana*, concurrent titrations as described above were made on control and experimental media. Colonies were counted and the means calculated and compared. If on experimental media colonies were not apparent at the end of five to seven days, or were too small for accurate counting, incubation was continued for two weeks.

The rickettsial composition of the colonies was routinely confirmed by the microscopic examination of stained impression-smear preparations, made by pressing a sterile 6-mm diameter cover-slip on the surface of the colonies, staining the smear by the methods of Giemsa or Giménez (1964), and mounting the cover-slip in balsam on a glass slide.

Blood samples from trench fever patients

Blood samples were drawn from three volunteer trench fever patients, heparinized, and stored at −60°C until used. Blood from H.F., who had been infected with the Osijek strain of *R. quintana* (Mooser et al., 1948) *via* infected louse faeces, was collected 27 days after the onset of the disease, when the patient was asymptomatic. Volunteers R.M. and S.M. had been infected with the Heliodoro strain originally isolated on basic medium from the blood of a trench fever patient in Mexico (Vinson et al.[1]) and passaged on this medium. Blood was drawn from R.M. 13 days after the onset of the disease, when the patient was asymptomatic, and from S. M. approximately 12 and 38 days following the insidious onset of afebrile disease. Xenodiagnosis on all three patients was positive.

To determine the approximate numbers of rickettsiae per millilitre of blood, the blood samples were diluted in sucrose-PG in log or half-log steps, and 0.05-ml volumes of each dilution were inoculated on each of five plates. After incubation, colonies on all plates were counted, and the mean number of colonies for each dilution was calculated. The calculation of the approximate numbers of rickettsiae per ml of blood, for which it was assumed that each rickettsia in the blood had formed a colony, was made as follows: the mean number of colonies on the five plates inoculated with a dilution was multiplied by 20 to give the mean number per ml at that dilution, and this figure was multiplied by the dilution factor to give the mean number per ml of blood. This calculation was repeated for each dilution used, and the mean of the figures obtained was calculated to give the approximate numbers of rickettsiae per ml of blood.

[1] Vinson, J. W., Varela, G. & Molina, C.—in preparation.

RESULTS

In vitro *propagation of* R. quintana *from blood samples from trench fever patients*

When heparinized blood or the blood clots of trench fever patients are streaked with a bacteriological loop on basic medium, colonies of *R. quintana* become visible in the simple stereoscopic microscope at 20× magnification after 12-14 day's incubation at 37°C in 5% CO_2 in air. The colonies are round, lenticular, translucent, mucoid, 65μ-200 μ in diameter, and grow only on the surface of the agar. Subculture is easily effected by cutting out agar blocks containing the colonies and emulsifying them in sucrose-PG in a vial containing glass beads or by scraping the colonies into sucrose-PG, with subsequent mechanical dispersion of the firmly adherent rickettsiae. On the first subculture on basic medium and thereafter the colonies become apparent three to five days after planting.

Numbers of R. quintana *in blood samples from trench fever patients*

The approximate numbers of rickettsiae circulating in the peripheral blood of three trench fever patients were estimated by titration on basic medium. In each case the etiology had been confirmed by positive xenodiagnosis. As stated above, volunteer H.S. had been infected with the Osijek strain and volunteers R.M. and S.M. had been infected with the Heliodoro (Mexican) strain of *R. quintana*, isolated and propagated exclusively on basic medium.

Results of the titrations are presented in Table 1. An estimated 5100 rickettsiae/ml circulated in the blood of H.F. on the 27th day after onset, when the

TABLE 1

ESTIMATED NUMBERS OF RICKETTSIAE IN BLOOD SAMPLES FROM THREE TRENCH FEVER PATIENTS

Volunteer patient [a]	Day after onset of disease	Titration of patients' blood [b]						Approx. No. of rickettsiae/ml blood [c]	Day on which colonies visible
		2×10^{-1}		10^{-1}		10^{-2}			
		Counts	Mean	Counts	Mean	Counts	Mean		
H.F.	27	ND		19, 22, 34, 34, 47	31	1, 1, 1, 2, 3	2	5 100	13
R.M.	13	18, 19, 20, 20, 22	20	10, 10, 11, 11, 19	12	ND		2 200	5
S.M.	~12	3, 5, 8, 11, 12	8	0, 2, 3, 3, 5	3	ND		700	5
	~38 [d]	3, 5, 5, 6, 15	7	2, 5, 5, 5, 8	5	ND		850	5

[a] Volunteer H.F. had been infected with a European (Osijek) strain of *R. quintana via* infectious louse faeces; Volunteers R.M. and S.M. with a Mexican (Heliodoro) strain, isolated and propagated exclusively *in vitro*.

[b] Log or half-log dilutions in sucrose-PG (see " Materials and Methods ") of the patient's blood were inoculated on 5 replicate plates in 0.05-ml volumes. The actual colony counts and their means are presented in the table. Dilutions not inoculated on plates are listed as not done (ND).

[c] Calculations, which assumed that each rickettsia in the blood formed a colony, were made as follows. The mean number of colonies at a given dilution was multiplied by 20 to equal the number of colonies/ml at that dilution. This figure multiplied by the dilution factor equals the number of colonies which would have been formed from 1 ml of undiluted blood. The mean of two such calculations made on each blood specimen is taken to equal the approximate number of rickettsiae/ml of blood—e.g., with Volunteer H.F.: $[(31 \times 20 \times 10) + (2 \times 20 \times 100)] \div 2 = 5\,100$.

[d] The irregularity of the counts is attributed to the presence of tiny blood clots in this specimen.

patient was free from symptoms. An estimated 2200 rickettsiae/ml circulated in the blood of R.M. on the 13th day after onset, when this patient also was asymptomatic. Twelve days after insidious onset of afebrile disease, an estimated 700 rickettsiae/ml circulated in the blood of S.M. and 38 days after onset, 850/ml. This latter figure is considered inaccurate because the blood contained small clots which caused an unequal distribution of rickettsiae, reflected in the erratic numbers of colonies in dilutions 5×10^{-1} and 10^{-1}.

Also given in the table are the days of incubation when the colonies were first visualized. Colonies from the blood of H.F. were visible after 13 day's incubation while colonies from the blood of R.M. and S.M., by contrast, were apparent after 5-6 days' incubation. As described in the preceding section, beginning with the first subculture after original isolation on basic medium, the rickettsiae multiply more rapidly to form detectable colonies about five days after planting. Formation of detectable colonies five to six days after inoculation with blood from volunteers R.M. and S.M., who were infected with a strain of *R. quintana* cultivated exclusively on basic medium, suggests that adaptation to the medium had not been lost by passage through human beings.

Effect of erythrocytes on in vitro *multiplication of* R. quintana

To determine the concentration of erythrocytes necessary for multiplication of *R. quintana*, media were prepared containing Difco blood-agar base, 6% serum, and 0, 0.01%, 0.1%, 1.0% or 4.0% erythrocytes. Where necessary, the volume was adjusted with salt K7G (see " Materials and Methods "). Results are presented in Table 2. The number of colonies growing on medium containing 1.0% erythrocytes was similar to the number on the control medium containing 4.0% erythrocytes, and the colonies were the same size (~200 μ). With 0.1% erythrocytes the number of colonies was diminished and they were smaller than those propagated on media with 1.0% and 4.0% erythrocytes. The number of colonies on media with 0.01% erythrocytes was reduced by about one log unit, as compared to the controls, and the colonies were so tiny, even after 13 days' incubation, that they were counted with difficulty. *R. quintana* did not multiply in the absence of erythrocytes. It is apparent that erythrocytes contain a factor, or factors, essential for multiplication of *R. quintana* under the conditions described, and that number and size of colonies depend, within defined limits, upon the concentration of erythrocytes in the medium.

TABLE 2

EFFECT OF ERYTHROCYTES ON *IN VITRO* MULTIPLICATION OF *R. QUINTANA*

Concentration of erythrocytes [a] in medium (Difco blood-agar base + 6 % serum [b] + erythrocytes)	*R. quintana* titration [c]			Comparative colony size
	10^{-4}	10^{-5}	10^{-6}	
0	0 [d]	0	0	
0.01 %	76	1	0	Extremely small
0.1 %	187	15	0	Small
1.0 %	368	32	0	~ 200 μ diameter
4.0 %	ND	50	0	~ 200 μ diameter

[a] Horse erythrocytes were washed 3 times in phosphate-buffered saline and haemolysed by freezing and thawing. Volumes and ionic concentrations were kept constant where required by the addition of salt K7G (see "Materials and Methods").

[b] Horse serum was inactivated at 56°C for 30 minutes.

[c] Log dilutions of *R. quintana* stock suspension from 10^{-1} to 10^{-6} in sucrose-PG (see "Materials and Methods") were inoculated on 3 replicate plates in 0.05-ml volumes. Colonies were counted after 7 days' incubation and rechecked after 13 days on plates with colonies smaller than normal. Means of the counts of 3 replicate plates at each dilution are presented in the table. Figures are given only for those dilutions producing 0-400 colonies per plate. Dilutions not inoculated on plates are listed as not done (ND).

[d] Colonies were not formed on plates inoculated with dilutions 10^{-1} to 10^{-3}.

Effect of erythrocytes treated with cold or heat on in vitro *multiplication of* R. quintana

The Fuller strain was titrated on basic medium in which had been incorporated erythrocytes (*a*) rapidly frozen to −60°C in a dry-ice/alcohol bath and thawed at 37°C, (*b*) slowly frozen to −30°C and thawed at room temperature, or (*c*) left untreated (apart from the usual haemolysis). Results are shown in Table 3, Experiment A. In Experiment B, Table 3, are presented results from an experiment in which the Guadalupe strain was titrated on media into which had been incorporated erythrocytes which had been (*a*) held at 56°C for 30 minutes, (*b*) held at 100°C (boiling water-bath) for 30 minutes, (*c*) autoclaved at 15 lf b/in² (1.05 kgf/cm²) for 15 minutes, or (*d*) left untreated. The titration was also performed on medium not containing erythrocytes.

It is again clear that *R. quintana* failed to multiply when erythrocytes were omitted from the medium. The growth-promoting factor (or factors) present in the erythrocytes was (or were) not destroyed by either method of freezing or thawing or by holding at 56°C or at 100°C for 30 minutes. Autoclaving the erythrocytes did not significantly reduce their activity.

TABLE 3

EFFECT OF ERYTHROCYTES TREATED WITH COLD OR HEAT ON *IN VITRO* MULTIPLICATION OF *R. QUINTANA*

Experiment	Treatment of 4 % concentration of erythrocytes in medium (Difco blood-agar base + 6 % serum [a], + erythrocytes)	*R. quintana* titration [b]			
		10^{-4}	10^{-5}	10^{-6}	10^{-7}
A (Fuller strain)	Untreated	TMTC	TMTC	27	3
	Rapidly frozen to −60°C; thawed at 37°C	TMTC	TMTC	32	2
	Slowly frozen to −30°C; thawed at room temperature	TMTC	TMTC	36	3
B (Guadalupe strain)	Untreated	ND	50	2	ND
	56°C for 30 min	ND	53	3	ND
	100°C for 30 min	209	30	4	ND
	Autoclaved at 1.05 kgf/cm² for 15 min	225	6	0	ND
	No erythrocytes	0 [c]	0	0	ND

[a] Horse serum was inactivated at 56°C for 30 minutes.

[b] Log dilutions of *R. quintana* stock suspension from 10^{-1} to 10^{-6} in sucrose-PG (see "Materials and Methods") were inoculated on 3 replicate plates in 0.05-ml volumes. Colonies were counted after 5-7 days' incubation. Means of the counts of 3 replicate plates are presented in the table. Figures are given only for dilutions producing 0-400 colonies per plate. More than 400 colonies per plate were considered too many to count (TMTC). Dilutions not inoculated on plates are listed as not done (ND).

[c] Colonies were not formed on plates inoculated with dilutions 10^{-1} to 10^{-3}.

Effect of haemoglobin on in vitro *multiplication of* R. quintana

To learn whether erythrocytes could be replaced by haemoglobin, the Fuller strain was titrated on media composed of Difco blood-agar base, 4 mg/ml of human haemoglobin and 0%, 5%, 10%, 15%, 20% or 33% serum and compared with a titration on basic medium. In another experiment titrations were made on media containing 20% serum and 0 mg, 2 mg, 4 mg, 6 mg, 8 mg or 10 mg haemoglobin per ml and compared with a titration made on control medium containing 20% serum and 4% erythrocytes.

From the results shown in Table 4, it would appear that at a haemoglobin concentration of 4 mg/ml, the number of colonies per dilution was a function of the concentration of serum in the medium. Multiplication of *R. quintana* did not occur on medium containing 4 mg haemoglobin per ml but no serum.

TABLE 4

EFFECT OF VARIOUS CONCENTRATIONS OF HAEMOGLOBIN AND SERUM ON *IN VITRO* MULTIPLICATION OF *R. QUINTANA*

Medium, i.e., Difco blood-agar base plus:		*R. quintana* titration [a]			
mg/hgb/ml [b]	% serum [c]	10^{-1}	10^{-2}	10^{-3}	10^{-4}
4	0	0	0	0	ND
4	5	395	5	4	ND
4	10	TMTC	136	16	ND
4	15	TMTC	201	23	ND
4	20	TMTC	363	35	ND
4	33	TMTC	378	52	ND
4% erythrocytes	6	ND	TMTC	75	9
0	20	0	0	0	ND
2	20	TMTC	135	14	ND
4	20	TMTC	141	12	ND
6	20	TMTC	159	8	ND
8	20	TMTC	196	17	ND
10	20	TMTC	135	12	ND
4% erythrocytes	20	ND	TMTC	50	4

[a] See Table 3, footnote *b*.
[b] Human hæmoglobin, twice recrystallized.
[c] Horse serum was inactivated at 56°C for 30 minutes.

At a serum concentration of 5%, the number of colonies in the several dilutions was irregular (five colonies at a dilution of 10^{-2} and four at 10^{-3}), an unusual finding, but at least 1-2 log fewer than those on the control medium. Increased numbers of colonies appeared on media containing increasing concentrations of serum. With a serum concentration of 33%, the number of colonies approximated to but did not equal the number of colonies counted on the control medium containing 6% serum and 4% erythrocytes.

On medium containing 20% serum the rickettsiae did not multiply in the absence of haemoglobin. A haemoglobin concentration of 2 mg/ml allowed multiplication of the rickettsiae but the number of colonies was at least 0.5 log fewer than the number of colonies counted on control medium containing 20% serum and 4% erythrocytes. Increasing the haemoglobin concentration from 2 mg/ml to 10 mg/ml did not result in an increase in the number of colonies.

It would appear that while haemoglobin cannot completely replace erythrocytes in the medium, it can, when combined with a high serum concentration, allow multiplication of *R. quintana* approximating to that observed on the basic medium.

Effect of blood serum on in vitro *multiplication of* R. quintana

R. quintana from a stock pool of the Fuller strain were titrated on media containing Difco blood-agar base, 4% erythrocytes, and either 6% inactivated horse serum, 6% sucrose-PG, or 6% salt K7G (see "Materials and Methods"). In an additional medium the serum was replaced by distilled water. In a related experiment, a different stock pool of the Fuller strain was titrated on media containing Difco blood-agar base, 4% erythrocytes, and 6%, 12% or 18% inactivated horse serum. The results (Table 5) show that the lack of serum in the medium did not prevent the multiplication of *R. quintana*. The numbers of colonies on media in which serum was replaced by the two solutions or by distilled water were lower than those on the control medium. While small, the differences are consistent. Increasing the serum concentration from 6% through 18% appeared to result in increasing numbers of colonies. Since this increase was noted only in the 10^{-3} dilution and not in the 10^{-4} dilution, however, it may present a chance artefact. Taken in conjunction with the data presented in Table 4, these results suggest that serum contains in small concentration a substance which promotes the multiplication of *R. quintana*.

TABLE 5

EFFECT OF SERUM ON *IN VITRO* MULTIPLICATION OF *R. QUINTANA*

Experiment [a]	Medium, i.e., Difco blood-agar base + 4% erythrocytes [b] plus:	*R. quintana* titration [c]			
		10^{-3}	10^{-4}	10^{-5}	10^{-6}
A	6% serum [d]	TMTC	274	33	1
	No serum	TMTC	106	5	1
	6% sucrose-PG [e]	TMTC	99	4	0
	6% salt K7G [e]	TMTC	91	10	1
B	6% serum [d]	50	4	ND	ND
	12% serum [d]	85	10	ND	ND
	18% serum [d]	103	5	ND	ND

[a] Different stock rickettsial suspensions of the Fuller strain were used in Experiments A and B.

[b] Horse erythrocytes were washed 3 times in phosphate-buffered saline and haemolysed by freezing and thawing.

[c] See Table 3, footnote *b*.

[d] Horse serum was inactivated at 56°C for 30 minutes.

[e] See " Materials and Methods ".

DISCUSSION

During the present interepidemic period of trench fever, cases of naturally occurring disease are seldom, if ever, reported, despite suggestive evidence that *R. quintana* is seeded in the human and louse populations in widely dispersed geographical areas. Aside from the classic endemic areas of trench fever in Poland (Kostrzewski, 1949) and the USSR (Zdrodovskij & Golinevich, 1960), cases of the infection or small circumscribed epidemics have been recorded in Ethiopia (Codeleoncini, 1946) and Tunisia (Sparrow, 1961). *R. quintana* is known to exist in Mexico (Varela, Fournier & Mooser, 1954) and its presence is suspected in mainland China (Liu & Landauer, 1952). Failure to recognize trench fever may be due to the difficulty of making a clinical diagnosis in the absence of an epidemic, the unavailability of a simple laboratory diagnostic test to establish the etiology, or the unfamiliarity of physicians with the disease. Other factors obscuring recognition might be the possible mildness of the disease in endemic areas, or its occurrence among population groups less well provided with medical services. Even if clinical trench fever be suspected, there is a paucity of laboratories capable of conducting the laborious xenodiagnosis heretofore required to substantiate the etiology of the infection.

We now have at our disposal a relatively simple method for the *in vitro* cultivation of *R. quintana*. The etiological agent of trench fever can be isolated with ease from the blood of patients. The protracted rickettsiaemia in trench fever, lasting for weeks or months or over a year following the primary clinical episode (Mohr & Weyer, 1964), makes for a relatively long period during which blood culture can be usefully employed. The occurrence of rickettsiaemia in the absence of clinical symptoms also suggests that *in vitro* cultivation of the rickettsiae from blood samples might become a useful epidemiological tool in heavily endemic areas of the disease.

In 1921 Sikora briefly reported the propagation of *R. pediculi* on human blood agar. After 20 days' incubation at 37°C, greyish-white colonies the size of pin-heads appeared. Growth was more profuse when inactivated blood was used. Subcultures succeeded on horse-blood agar but not on rabbit-blood agar. These suggestive studies were apparently not pursued, and Sikora was under the impression, current at that time, that *R. pediculi* was a harmless parasite of lice. Although the taxonomic position of *R. pediculi* has been questioned, most authorities consider it synonymous with *R. quintana* (Wolbach et al., 1922; Kostrzewski, 1949; Mooser, 1959) and it has been so classified by Philip (1953).

Pshenichnov (1957) has reported the propagation *in vitro* of *R. quintana* anaerobically in a medium composed of unstated proportions of milk, egg yolk, and defibrinated human blood. On the solid form of this medium, *R. quintana* was stated to grow as " minute greyish colonies, resembling cauliflower heads ". Further details of cultivation were revealed in a more recent publication (Pshenichnov et al., 1965). Addition to the milk/egg-yolk/blood medium of 10%-20% medium No. 199, a vitamin complex, or guinea-pig-organ extracts, and incubation in the presence of air were reported to increase the number of successful cultures from 5.7% to 60%-90%. Under these conditions the micro-organisms multiplied slowly, reaching their maximum growth in about 21 days. Colonies grew down into the medium, and it was difficult to separate them from the surface. The authors concluded that *R. quintana* was a facultative anaerobe. Complement-fixing antigens prepared from the micro-organisms cultivated *in vitro* were stated to react positively with convalescent sera from trench fever patients.

The absence of data in the available published reports precludes evaluation of these studies and valid comparison of their results with those of the

present writer. In addition, the authors did not state whether or not their strains cultivated *in vitro* had been identified by the classic procedures for identification of micro-organisms presumed to be *R. quintana*, i.e., induction of clinical trench fever in volunteers following inoculation of the suspected micro-organisms and confirmation of the etiology by positive xenodiagnosis with lice proved to be free of rickettsia-like micro-organisms.

Certain differences, however, can be noted between the cultural characteristics of *R. quintana* described by Pshenichnov et al. (1965) and those observed by the present writer. Under the cultural conditions described in the present paper, *R. quintana* forms detectable micro-colonies about the 12th day on original *in vitro* isolation from patients' blood, and in from three to five days after planting on subsequent subculture. The colonies are round, lenticular, translucent, and mucoid, and they grow only on the surface of the agar. In our hands the rickettsiae have failed to grow under anaerobic conditions or in the absence of added CO_2. The differences in the rate of growth and colonial morphology can perhaps be ascribed to differences in cultural methods, but the differences in gas-tension requirements are less easily resolved. The photomicrographs of *R. quintana* grown in liquid medium by Pshenichnov et al., however, show the tight clusters of micro-organisms noted by Vinson & Fuller (1961) within cells cultivated *in vitro* and in colonies examined by Ito & Vinson (1965) in the electron microscope.

The present studies clearly show that erythrocytes contain a factor, or factors, essential for multiplication of *R. quintana*. The essential factor or factors in erythrocytes was (or were) not destroyed by freezing to $-60°C$ or $-30°C$, or by heating at 56°C or 100°C for 30 minutes, or by autoclaving at 15 lbf/in² (1.05 kgf/cm²) for 15 minutes. This heat stability is reminiscent of that of the haeme portion of the haemoglobin molecule. Haemoglobin alone could not replace erythrocytes in the medium, but a combination of haemoglobin with high concentrations of serum allowed multiplication of *R. quintana*. It seems likely that haemoglobin, or some substance closely associated with it and not eliminated by purification procedures, may be one of the factors in erythrocytes essential for multiplication of *R. quintana*.

The results suggest that serum contains a substance capable of promoting the growth of the rickettsiae. The effect of this substance was only slightly apparent when erythrocytes were present in the media but was readily demonstrable when human haemoglobin was substituted for erythrocytes. The identity of the serum factor, or factors—whether a normal serum component or a substance liberated from erythrocytes—was not determined.

The dependence of *R. quintana* on components of blood for multiplication is not surprising in view of its exclusively extracellular growth in the lumen of the louse gut, an environment containing predominantly blood and its digested products. Although the sites of multiplication of *R. quintana* in the human body are unknown, fully infectious rickettsiae circulate in the blood stream, and several of the physical manifestations of the disease suggest an involvement of the rickettsiae with blood-forming organs.

During studies on the propagation of *R. quintana* in cell culture (Vinson & Fuller, 1961) the medium used to support cell growth contained serum from human blood which had been defibrinated with glass beads and was consequently somewhat haemolysed. When clear horse serum obtained from clotted blood was substituted for human haemolysed serum, the majority of the rickettsiae disappeared from the cells within 24 hours and by 48 hours rickettsiae could no longer be visualized in stained smears of cells or medium. While this unpublished observation was not further investigated, it suggested that even in an intracellular environment, components of erythrocytes might have been essential for multiplication.

The favourable influence of blood on the multiplication of other rickettsiae has been noted by several authors. Mooser, Varela & Pilz (1934) achieved heavy growth of *R. prowazekii* in the peritoneal cells of the rat by the daily intraperitoneal injection of guinea-pig blood. These authors stated that "*R. prowazekii* is able to develop in the mammal as well as in the insect only within those cells which come into constant contact with fresh blood". Travassos (1938) subsequently reported the maintenance of *R. rickettsii* in serial passage in white rats by intraperitoneal inoculations of the rats with guinea-pig blood, an observation confirmed by Harshman, Johnson and Price (1958) and Velasco & Varela (1963).

The blood factor or factors reported by Harshman et al. to enhance growth of *R. rickettsii* in the white rat resided exclusively in the erythrocytes and could not be replaced by bovine haemoglobin. Activity was not lost by freeze-thaw procedures but, in contradistinction to the factor or factors in erythrocytes found to promote multiplication of *R. quintana*,

activity was readily destroyed by heating to 100°C for five minutes or by lysis of the erythrocytes with distilled water.

Criteria established for evaluating the effect of media components on multiplication of *R. quintana* were size and numbers of colonies, as compared with the controls. Colony size at a given time during incubation appeared to reflect the rate of multiplication of the rickettsiae. In some instances incubation of experimental plates had to be prolonged until colonies became sufficiently large for accurate counting. When colonies were not formed on experimental media it was clear that the media lacked a substance or substances, e.g., erythrocytes, without which the rickettsiae could not multiply. Less amenable to precise interpretation are results of experiments in which fewer numbers of colonies appeared on experimental media than on basic medium. Reduction in the number of colonies implied that the medium lacked a factor permitting multiplication of *all* rickettsiae inoculated on to that medium. The appearance of colonies, however, even in diminished numbers, would seem to reflect individual differences among the rickettsiae either in their metabolic state at the moment of inoculation or else in their varying potentials for responding to changed nutritional circumstances.

These observations assume practical importance in the propagation of *R. quintana* from the blood of trench fever patients as a diagnostic procedure. While it is likely that not all rickettsiae circulating in the blood are capable of multiplying on the medium devised, it is apparent that reduction of the numbers which can multiply by 0.5 log to 2.0 log, by the use of suboptimal cultural conditions, can diminish the chances for successful isolation.

In view of the similarities in the metabolic activities of *R. quintana* and the other rickettsiae (Huang & Weiss, 1965), it is hoped that continued studies on the nutritional requirements of *R. quintana* may provide clues for the elucidation of the nutritional requirements of the obligate intracellular rickettsiae.

ACKNOWLEDGEMENT

The author acknowledges with pleasure the technical assistance of Mrs Sylvia Sawyer and Miss Ann Shortell.

RÉSUMÉ

La fièvre des tranchées reste endémique en de nombreuses régions; son diagnostic est difficile cliniquement en dehors des épidémies et, au laboratoire, ne pouvait être établi que par xénodiagnostic. La culture de son agent étiologique *Rickettsia quintana* avait été obtenue à 35°, sur milieu de base gélose au sang Difco, enrichi de 10% de sang défibriné d'homme ou de cheval. Mais, si ce milieu convenait aux souches européennes de *R. quintana*, il ne permettait pas de cultiver les souches mexicaines.

L'auteur a remplacé le sang défibriné par 6% de sérum de cheval inactivé et 4% d'hématies de cheval lavées. L'emploi d'hématies hémolysées a donné un milieu transparent permettant de mieux voir les colonies microscopiques de *R. quintana*, dont la croissance est accélérée à 37°. Le sang de lapin peut remplacer celui de cheval ou d'homme. Les colonies ne se développent qu'en atmosphère contenant 5% de CO^2 et n'apparaissent pas en anaérobiose.

Le sang du malade est directement ensemencé sur le milieu. Les colonies deviennent visibles au microscope stéréoscopique, après 12-14 jours d'incubation; elles sont rondes, lenticulaires, transparentes, mucoïdes, d'un diamètre de 65-200 μ; elles ne poussent que sur la surface de la gélose. Dès le premier repiquage sur le même milieu, elles apparaissent en 3-5 jours. La vitesse de croissance est la même lorsqu'on cultive le sang de volontaires infectés avec des souches entretenues sur le milieu de base. Il semble donc exister une adaptation à ce milieu qui ne se perd pas par passage sur l'homme. Les érythrocytes contiennent un ou des facteurs essentiels à la multiplication de *R. quintana*; stable au froid et à la chaleur, il correspond peut-être à la fraction hème de l'hémoglobine; le sérum sanguin facilite également la croissance.

Cette méthode de culture fournit un moyen relativement simple d'isolement de *R. quintana* pour le diagnostic ou la préparation d'antigènes. Elle permet de calculer le nombre de rickettsies circulant dans le sang périphérique du malade. Elle est utilisable pendant une longue période, en raison de la rickettsémie prolongée de la fièvre des tranchées; elle a un intérêt épidémiologique certain, de nombreux sujets apparemment sains présentant une rickettsémie dans les régions d'endémie.

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