Q FEVER — PHASE VARIATION OF *C. BURNETII* AND ITS RELATION TO PATHOGENICITY AND IMMUNITY

MANUEL R. PINTO
Instituto de Medicina Tropical — Lisbon, Portugal

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This report was prepared in collaboration with Prof. Fernando Fonseca and Drs. António P. Franco and J. Carvalho de Sousa and, in part, it is based on results obtained during experimental work carried out under the orientation of Prof. Fonseca.

We wish to discuss three aspects of Q fever — clinical, immunological and epidemiological — which are linked to a certain extent.

From the clinical point of view we maintain the classification of Q fever that we presented in January 1949: *a*) febrile illness without localization; *b*) febrile illness with pulmonary or pleuropulmonary localization; *c*) illness progressing in a form that we had not seen referred to in literature, as we wrote in 1949, with the agent attacking at different points in the organism. Medical literature is rich to-day in descriptions of clinical cases belonging to group *c*) and the most varied localizations have been presented with the corresponding symptomatology. Already at that time we were struck by the prolonged course of the illness in some patients in group *c*), with successive localizations of the agent, such as we observe in the cyclic diseases as, for example, brucellosis.

By that time, as therapy, we had only penicillin, streptomycin and para-aminobenzoic acid. One of the patients became ill in April 1948 with the usual symptomatology (cephaleas, orbitary pains, temperature, epistaxis, haemoptoic expectoration and negative radio-

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graphic examination of the thorax). Some days after mild jaundice and urine with biliary pigments — hepatitis. Progressive fall in temperature and feeling of being in convalescence. Without entering in apyrexia there was a worsening of the disease on the 15th May with, at first, an increase in the volume of the abdomen with ascites, and later a pleuro-pulmonary process of the left base and of the right pleura. From 25 May to 1 June a daily injection of 1 g of streptomycin was given and twice an intra-peritoneal injection of the same drug, without benefit. Improvement on treatment with para-aminobenzoic acid and on 18 June 1948 there were no signs of the illness, but on radiographic examination of the thorax there were still slight signs of pulmonary affection of the left base. Two months of illness with hepatic, peritoneal and pleuro-pulmonary localizations.

Cases of chronic evolution, such as that we have just referred to, now appear more frequently, although still rarely, and we wish to draw attention to certain of their particularities. The first is that of the cyclic evolution, corresponding to successive localizations in different organs, with or without intermediary periods of apyrexia. The second is the little or no efficiency of the therapy which, to-day we almost consider specific. The various tetracyclines and chloramphenicol are generally effective against Q fever as long as they are used at high doses. However their effect is merely rickettsiostatic, and therefore for the cure, a good antibody response is required from the patient to the aggression of Coxiella. Regarding the Q fever agent, it is possible that a better knowledge of the antigenic variation which we shall discuss later, and of the antibody response, may explain the chronic forms of the disease and the failure of the treatment. According to Abinanti and Marmion (Abinanti, F. R., and Marmion, R. P. — *Am. J. of Hyg.*, **66**: 173, 1957): «The recognition of the protective effect of antibody directed against the phase 1, of 'Wild' from of *C. burnetii* may be of importance in assessing or understanding the immune response to Q fever vaccines, or other immunological problems, such as the chronic or relapsing human case of Q fever, in which there may perhaps be a failure to form antibody against this antigen». The case we wish to present, like the first, had successive localizations which prolonged the illness unusually; it shows the possibility that such evolution may depend on the antibody response to Phase 1 antigens and moreover it may

explain the failure of the specific therapy by the deficiency of antibodies corresponding to this antigen.

Mr. J.A.S.P. is a medical doctor and has a farm in Trás-os-Montes [1]. He fell ill with a high temperature (40°C), cephaleas and dizziness on 5 February 1957, shortly after being at his farm, where he has livestock and where he drank milk and ate fresh cheese. There is brucellosis in the region. For three days he had anti-influenza therapy without result (Synergistin 2 ampules daily, Ricolon and Afragon) and to the previous symptomatology, anorexia, insomnia and slight photophobia were added. With temperatures between 38° and 39° he took Tetracyn from 8 to 17 February, together with Vitamine C, Complex B and Cortone, from 18 to 24 of the same month Chloramphenicol, on the 25 and 26 Iloticine, and on 27 and 28 February and 1 and 2 March Nivaquina Specia. The temperature remained unaffected by these successive treatments. The patient lost 14 kg weight and an X-ray examinations on 12 February revealed the existence in the right pulmonary base of a small focus in which there was noted a slight lessening of permeability and reinforcement of the reticulum, alterations which disappeared on further examination on the 23rd of the same month. No treatment from 3 to 6 March, in order to repeat analyses. On the objective examination we found only a right epididimytis which the patient attributed to aggravation of an orchiepididimytis suffered 30 years before. Another attempt of therapy with Aureomycin for 3 days, without result . Temperatures between 37° and 38°, with, at times, peaks at 39°.

The negative results of laboratory examinations (due to difficulties in obtaining Q fever antigen, the result of the complement fixation test for Q fever was known only later) led to the diagnosis of tuberculous epididimytis and a therapy with 1 g of Streptomycin daily and 8 tablets of Dipisac daily, Vitamins and 1 tablet of 0.25 g of pyramidon when temperature rose was iniciated on 23 March, and continuing with the same drugs and pyramidon, he began to get up, slep better and had a better appetite. However he felt dizziness while

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[1] This history were partly supplied by Dr. A. Cotta and Prof. Júlio M. Vaz, who followed the progress of the patient in Oporto and by Dr. Neves Tavares who treated him in Lisbon. We thank these colleagues for their kindness.

walking and tachycardia at 100-115. Streptomycin was administred on alternate days. From 2 to 14 April he maintained sub-febril temperatures and from 15 to 22 of the same month, despite the pyramidon, a new febrile period appeared with pains in the body, aggravation of the epididymal process which was tending to become attenuated and increase of the erythrocite sedimentation rate to 75 mm in the first hour. From 23 April to 6 May he remained afebrile, without pyramidon and, continuing with streptomycin and Dipasic, he suffered from a new febrile attack, with wide oscillations of temperatures from 37° to 38.5°. He turned to pyramidon again and the febril temperatures disappeared from 17 May up to 2 June. On 17 May he stoped with the anti-tuberculous treatment. The recommencement of the epididymal process after such a prolonged treatment, and the negativity of the laboratory examinations (cultures in Lowenstein and inoculations in guinea-pigs of urine and fluid from prostatic expression) led us to exclude the tuberculous aetiology and to valorize the C.F. test for Q fever. The febrile period which begun on the 3 June, lasted until 10 June, disappearing after Neo-Acromycin administration (1 g daily), which was begun on the 6th and continued until the 26th of the same month. The inflammatory symptoms of the epididymus disappeared rapidly and the patient entered a period of convalescence and latter has taken up all his activities.

Like other infectious diseases Q fever can take a chronic evolution with relapses due to new localizations, corresponding to a deficit of the immunity process, linked probably to a weak response to the Phase 1 antigens as it is suggested by Stoker's experience (Stoker, M. G. P., and Fiset, P. — *Canad. J. Microbiol.*, **2**: 310, 1956).

The laboratory examinations carried out during the five months of Mr. J.A.S.P's. illness led to the exclusion of the most frequent infectious diseases in the regions he usually lives in: typhoid fever, brucellosis, etc. and tuberculosis. The intra-peritoneal inoculation of the patient's blood in mice and guinea-pigs in attempts to isolate *C. burnetii* were unsuccessful, but we observed the patient a month after his falling ill and after prolonged treatment with tetracyclines and chloramphenicol.

Let us now analyse the results of the C.F. test for Q fever. We have used as antigen the Nine-Mile strain kindly supplied by

Dr. H. R. Cox, and for the C.F. test we followed the conventional technique using 2 units of antigen and 2 units of complement.

The results obtained were as follows: on 23 April 1957 positive at 1/2560, on 16 May positive at 1/640, on 16 June positive at 1/2560, and on 6 May 1958 positive at 1/80.

Later we had the opportunity of re-testing some of the samples of the patient's serum using as antigen the Christie strain in Phase 1, which was kindly sent to us by Dr. M. G. P. Stoker. The results obtained were the following: on 16 May 1957 positive at 1/80 and on 6 May 1958 still positive at 1/40.

The appearence of antibodies against Phase 1 antigens in the patient's serum at a titre that we can consider high coincided with progress of the patient towards the cure. The hypothesis that the immunity response to the antigens of Phase 1 may be of importance in the fight of the organism to overcome the disease seems to be supported by these observations.

In cases of more rapid evolution without localization, in which antibodies for Phase 1 are rarely detected, it is possible that they exist but in quantities that they are not revealed by the conventional C.F. test as suggested by Abinanti and Marmion (*Am. J. Hyg.*, **66**: 173, 1957). However these authors were able to demonstrate the existence of small quantities of protective antibodies in vaccinated subjects and Stoker verified that the serum of one of his patients with a high titer of protective antibodies gave a positive C.F. test at a high titre with antigen of Phase 1. If there is a correlation between the presence of serum-protective antibodies and the presence of antibodies to Phase 1 as these authors suggest, it seems possible to admit that the existence of antibodies against Phase 1, even at a low titre, may play an important role in the evolution towards the cure of these cases without localization.

This hypothesis needs to be worked out with experiments in which strains in Phase 1 and Phase 2 will be used and in which other serological methods will be employed, possibly based on techniques of tissue culture.

Another aspect of the problem that we consider of interest to investigate is the sensitivity of these microparasites to chloramphenicol and tetracyclines, in relation to the phase variation. It is possible to think that when C. *burnetii* is in Phase 1, that is to say,

with all its antigenic equipment, it may show a greater sensitivity to these antibiotics.

The appearance of the antibodies against Phase 1 is more delayed than those to Phase 2, even when the animals are inoculated with strains in Phase 1. The experiments by Stoker *et al.* and by Fiset, quoted later, suggest that although the animals are inoculated with Coxiella in Phase 1, a great part of these microparasites lose surface antigens during the first phases of their struggle against the host and expose their deeper antigens — Fiset's «good antigens» — which give rise to a prompt response in antibodies against Phase 2. The later appearence of antibodies to Phase 1 could correspond to a process of selection of the picroparasite in Phase 1 and its subsequent predominance, which could arise due to the presence of antibodies against Phase 2 which reach a high titre. In fact Stoker and Fiset were able to recuperate Coxiella in Phase 1 from guinea-pigs inoculated with Phase 2 microorganisms when these animals began to produce antibodies against phase 2 antigens.

In these circumstances and if the sensitivity of the Coxiella in Phase 1 is more marked in relation to antibiotics it is possible to admit that the immunizing mechanism could be linked with a better activity of the antibiotics in order to bring about the cure of the disease specially in the cases of more prolonged evolution.

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From the immunological point of view we wish to call the attention to the importance of the discovery of Stoker and co-workers on the antigenic variation of *C. burnetii* and its repercussion on the interpretation of the immunizing processes in the course of Q fever in man and animals.

In the literature there are no references to the observation of marked antigenic variation among microorganisms of the genus Rickettsia, although the strains may differ considerably when compared by complement fixation tests. Among the strains of *C. burnetii* there are no antigenic relations with the Rickettsia, but there exist great differences among the strains as to their reacting capacity in complement fixation tests.

Strongly reactive strains have been described such as the Henzerling, the Nine-Mile and others which are poor in their

complement fixation capacity in the presence of anti-sera (Robbins, F. C., *et al.* — *Am. J. Hyg.*, **44**: 5, 1946; Topping, N. H., *et al.* — *Am. J. Hyg.*, **44**: 173, 1946; Smadel, J. E., *et al.* — *Am. J. Hyg.*, **47**: 1948; Pinto, M. R. — *An. Inst. Med. Trop.*, **9**: 5, 1952; Berge, T. O., and Lennette, E. H. — *Am. J. Hyg.*, **57**: 144, 1953).

In 1953 Stoker had the merit of throwing light on the behaviour of a strain of *Coxiella burnetii* isolated by MacCallum *et al.* (MacCallum, F. O., *et. al.* — *Lancet*, **2**: 1026, 1949) from a human case of Q fever which when used as antigen was not capable of reacting with antibodies of the patient's serum, antibodies which were shown by the Henzerling strain. However that same strain (Christie strain), after 5 egg passages began to give antigens which showed up the antibodies in homologous serum. The same author showed further that the phenomenon was due to antigenic variation.

In later work of his Department of Pathology at the University of Cambridge and in collaboration with Fiset he demonstrated that strains of *C. burnetii* isolated from man, animals and arthropod vectors, generaly fail to react in the complement fixation test with sera from Q fever convalescent patients. Only after serial passages in the yolk sac do these strains acquire reactive capacity. The work of Fiset (Ph. D. Thesis, University of Cambridge, 1955) threw light on some aspects of the antigenic variation of strains of *C. burnetii* (Nine Mile, Christie and others isolated from ticks cow's milk and sheep placenta). The strains tested, when in Phase 1 (the «wild» form), as a rule, were non-reactive with homologous sera in the complement fixation tests and only acquired reactive capacity after several egg passages as mentioned above.

The strains that had been passed in animals, such as a Nine Mile line already passed 308 times in guinea-pigs, retained the characteristics of Phase 1, only acquiring the characteristics of Phase 2 after 8 egg passages. Inversely Fiset showed that it is possible to induce the reversion from Phase 2 to Phase 1 by passages in animals.

The immunity response of laboratory animals, specially guinea-pigs and rabbits, was carefully investigated after infection of the animals with one or other variant and also after vaccination with killed suspensions of *Coxiella* in Phase 1 and in Phase 2. The behaviour of the animals after infections is similar, the antibodies

to Phase 2 appearing during the second week after infection and those to Phase 1 only later, after the fifth week, maintaining high titres during the long experimental period.

The antibodies response after vaccination is similar and comparable to that observed after experimental infection, with the exception of vaccination with Phase 2 which only brought about, as would be excepted, antibodies to Phase 2. However the experiments on rabbits showed results that seemed paradoxal at first. The rabbits vaccinated with the Nine Mile strain in Phase 2 produced antibodies against Phase 1, but Fiset interpreted this finding as an indication of a mixed population.

Some aspects of the aggressivity of the *Coxiella* and of the antibody response during the disease have interested our group.

We have been working with a Portuguese strain «Nobre Marques» (N. M.) which has already been analysed by one of us (Pinto, M. R. — *An. Inst. Med. Trop.*, **9**: 5, 1952) and which was maintained in eggs (30 passages) and later lyophilized.

This strain which behaves like a good Phase 2 antigen in the complement fixation test was studied from the point of view of its aggressivity to animals and men. When it is inoculated in guinea-pigs it produces an experimental disease accompanied by the production of antibodies against Phase 2 and later against Phase 1 in a similar way to that observed by the authors quoted (Fig. 1).

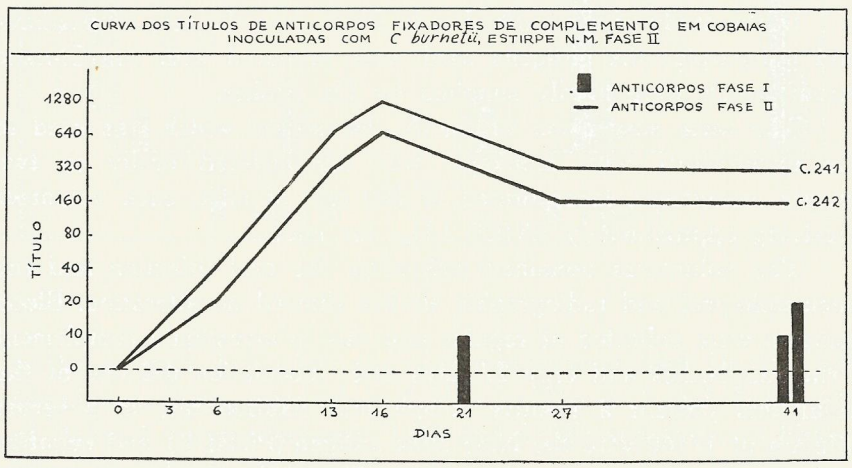


Figure 1

However this N. M. strain in subsequent passages from guinea-pig to guinea-pig and specially from mouse to mouse did not show an increase of its aggressivity towards these animals, but on the contrary a more benign illness was observed and a gradual loss of the power to provoke splenomegaly in guinea-pigs and mice. This aspect of the aggressivity probably correlated with the antigenic variation needs further studies before conclusions can be drawn.

The inoculation of monkeys (Cercopithecus) by subcutaneous route with 0.2 cm^3 of 10 percent suspension of *Coxiella* in Phase 2 containing approximately 13 500 LD_{50} for mice intraperitoneally as calculated by the method of Reed and Muench (*Am. J. Hyg.*, **27**: 493, 1938) according to the appearance of splenomegaly, or 200 000 $TCID_{50}$ calculated from the microscopic observation of the tissue culture sediments of the tubes used in the titration, induced febrile disease with signs of prostration in three of the animals inoculated. The evolution of the antibodies is expressed in Fig. 2.

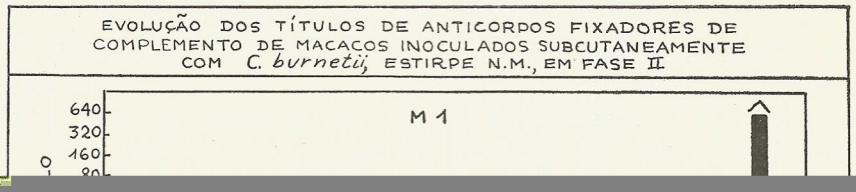
The oral administration of 0.5 cm^3 of the same suspension of *Coxiella* in Phase 2 in Hanks' solution and mixed with 4.5 cm^3 of milk, suspension that contained approximately 34 000 LD_{50} for mice, produced a more benign form of the illness in each of the three monkeys inoculated and the curves of the appearance of antibodies are summarised in Fig. 3. The inoculation in guinea-pigs of blood taken from monkeys of one or the other group, collected during the pyrexia allowed the recuperation of N. M. strain.

In the study of the antibodies to Phase 1 in the sera of monkeys and guinea-pigs the antigens used were prepared with the Christie strain which were kindly supplied by Dr. Stoker.

The same suspension of the N. M. strain, which was used in the experiments with monkeys, was administered orally to two women volunteers, incorporated in 200 cm^3 of milk, each volunteer receiving approximately 68 000 LD_{50} for mice.

The volunteers remained well after the oral infection and the haematological and radiographic studies showed no alteration. Blood samples were collected at regular intervals to investigate complement fixing antibodies and only 27 days after inoculation did one of the volunteers present a positive complement fixation test at a serum dilution of 1/160 with the Nine Mile antigen (Phase 2) and negative with the Christie antigen (Phase 1). The C.F. test remained posi-

tive at 1/80 on 30 August 1958 with a Phase 2 antigen. The intraperitoneal inoculation in guinea-pigs of blood collected from a volunteer one week after infection, did not provoke a febrile disease



By the results obtained in these experiments carried out with a strain of *Coxiella* in Phase 2 it can be thought that *Coxiella* in Phase 2 do not possess the same aggressive capacity as those in

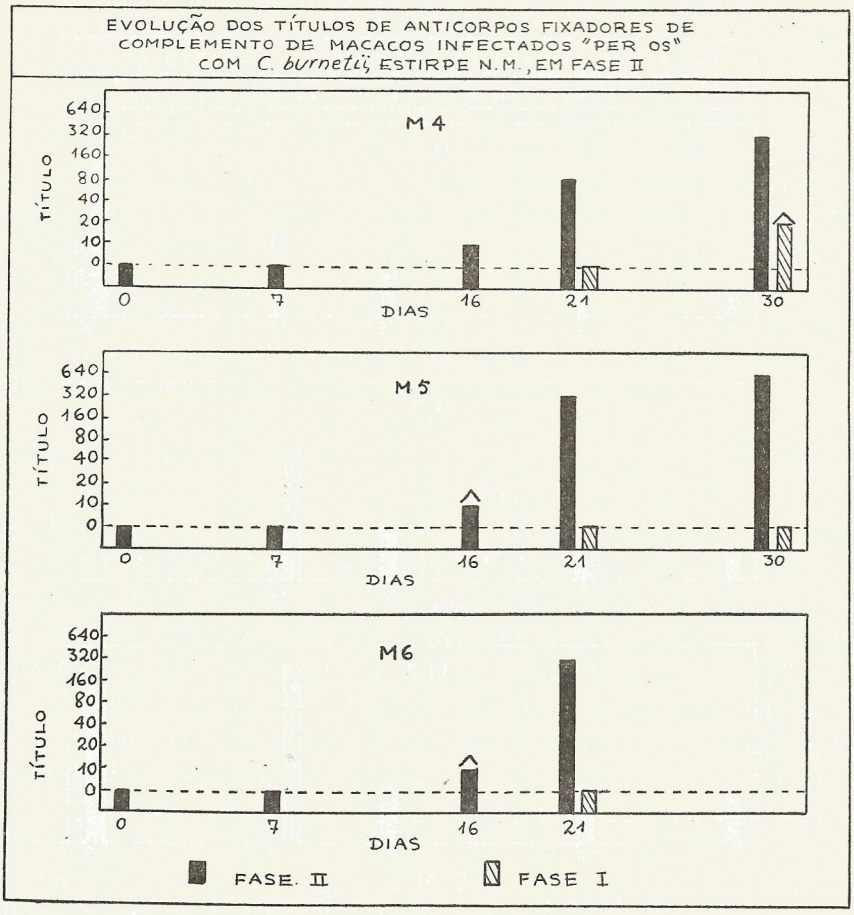


Figure 3

Phase 1, but light needs to be thrown on this problem. It is possible that laboratory infections described in medical literature, some of them taking a serious evolution, may have been provoked by *Coxiella* in Phase 1, since some of them occurred when workers were beginning their experiments in eggs and on the other and, it is not referred as

far as we know, infections in laboratories where antigens are prepared from *Coxiella* already passed may times in the yolk sac.

Another aspect of the biology of *Coxiella* which we were interested in investigating was the possibility of obtaining in tissue culture and in the presence of antibodies to Phase 2 the variation of *C. burnetii* from Phase 2 to Phase 1.

Fiset (loc. cit.) succeeded in obtaining this reversion in the yolk sac of the developing chick embryo, using a strain in Phase 2 and making its culture in the presence of antibodies to Phase 2.

To carry out our experiments we used two cellular lines, cells prepared from chick embryos with 11 to 13 days of evolution and cells of mouse embryos prepared from embryos collected in pregnant females at the end of gestation.

The technique of preparation of the tissue culture was similar for both cellular lines. After trypsinization of the small fragments of tissues, the cell suspensions were suspended in a medium generally consisting of 60 percent of Hank's solution with 30 percent bovine amniotic fluid or 30 percent of Difco 199 medium and 10 percent calf inactivated serum. Antibiotics were added. The media differed essentially in the alternate use of 199 medium or bovine amniotic fluid, the better cell growth being obtained when the 199 medium was used.

The cell suspensions were prepared to contain approximately 1 500 000 cells per cubic centimeter and were distributed in tubes or bottles which were kept stationary. The cellular growth was abundant after 24-48 hours of incubation.

The infection of the tubes or the bottles was made immediately after the seeding of the cells using a standard inoculum consisting of a 10 percent suspension in Hank's solution of yolk sacs obtained from eggs that died between the 4th and 5th day after infection, showing abundant growth of *Coxiella*.

The infection of the cells was very rapid, being possible to observe the microparasite in the citoplasma of the cells four hours after infection and small microcolonies five hours after infection. These microcolonies increased rapidly in size and 10 hours after infection they invaded the cytoplasma of the parasitised cells (Figs. 4 and 5).

From these experiments it was possible to conclude that the N. M. strain of *Coxiella burnetii* grows very easely and abundantly

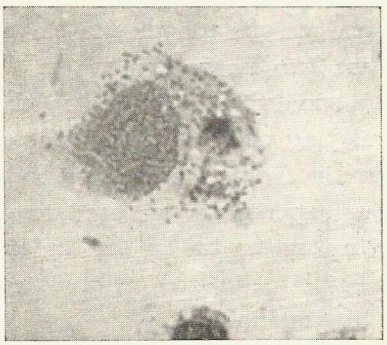


Fig. 4 — Infected cell 5 hours after infection with *C. burnetii*

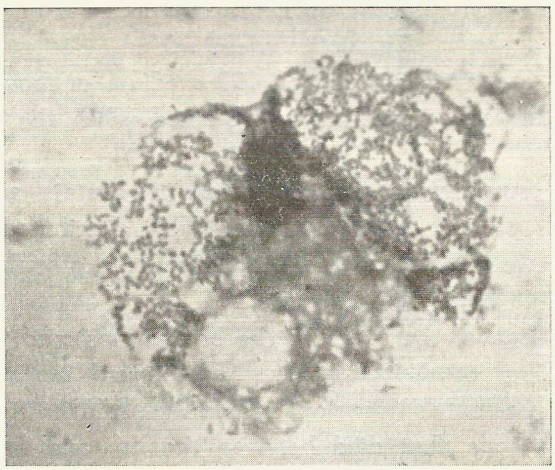


Fig. 5 — Infected cell 10 hours after infection with *C. burnetii*

in tissue cultures of chick embryo or mouse embryo (Fig. 6) presenting the characteristic morphology. No marked alterations in the morphology of the infected cells was observed.

The very satisfactory frowth of *Coxiella burnetii* in these tissue cultures gave the possibility of preparing good antigens for the C.F. test. After 5-6 days of incubation the fluids from the bottles were collected together with the cellular debris and after centrifugation, resuspension in formol-saline and treatment by ether, the water phase was very rich in *Coxiella*. After standardization of the suspensions, the antigens were titrated and then used in the C.F. tests with good results. It is worth to call the attention to the easy preparation of the antigens obtained from infected chick embryo tissue cultures.

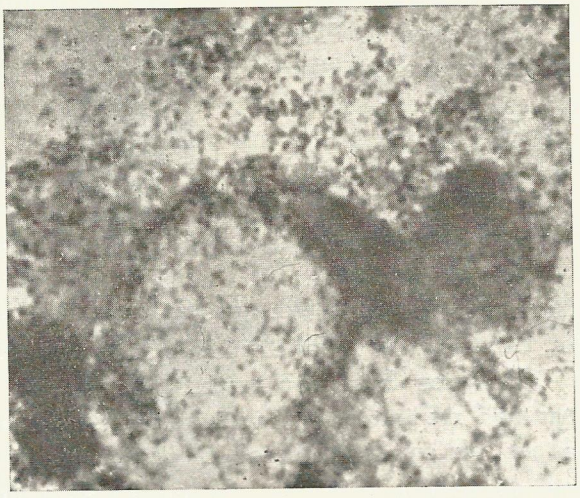


Fig. 6 — A culture of *C. burnetii* in chick embryo tissue culture

The reversion of *Coxiella burnetii* in Phase 2 to Phase 1 was attempted in mouse embryo and chick embryo tissue cultures. For this purpose four serial passages of the N. M. strain of *Coxiella burnetii* were made in tissue cultures, adding to the fluids of the cultures guinea-pig immune serum with a C.F. titre higher than 1/640 to Phase 2. The amount of serum added was 0,1 cm^3 of a 1/50 dilution of the guinea-pig serum per tube in the first passage, 0.1 cm^3 of a 1/30 dilution of the same serum in the 2nd passage and 0.1 cm^3 of a 1/20 dilution in the 3rd and 4th passage.

The growth of the *Coxiella* in the first two passages was very poor as compared with the control tubes without immune serum but on the third and fourth passages the growth became abundant again.

The fluids of the cultures of the 4th passage containing cellular fragments in suspension were withdrawn after 5-6 days of incubation and antigens were prepared from them.

The antigens thus obtained were studied in a chessboard titration with human and guinea-pig sera previously tested with Phase 2 antigen (Nine Mile) and Phase 1 antigen (Christie). The results are summarized in Fig. 7 and Table 1 and show that the culture of *C. burnetii* in Phase 2 reverted to Phase 1 as desired. The antigens lost their capacity to detect the anti-Phase 2 antibodies present in guinea-pig C 241 serum.

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As for the epidemiology of Q fever, a subject which at this Congresses will be dealt with by Dr. E. H. Lennette, it seems to be possible to maintain the same point of view that we referred in 1951 (*Clin. Contemp.*, v, 1, Janeiro 1951, and *Gazeta Méd. Port.*, iv, 3, 1951): Contagion from man to man is rare and the disease is acquired through respiratory or digestive route. We attributed to milk an important role as the source of infection of many cases of Q fever observed in Lisbon. However, the recent data presented by Dr. Lennette shows the great importance of the respiratory route.

Epizootics and epidemics of Q fever go hand in hand and the latter, which is a shadow of the former depends entirely on it; with or without a reservoir in wild mammals, since vectors transmit the *Coxiella* to offspring, the continuity of epizootics is assured; the infection of the young is admited not only transplacentally (*Gazeta Méd. Port.*, iv, 3, 1951) but also by the mother's milk (Caminopetros cit. by Babudieri in *Recenti Progr. in Med.*, **9**, No. 3, 1950) and the immunity of the adult animals would lead to the progressively disappearance of Q fever; Q fever would spread through periods of activity, followed by others in which the disease would almost disappear, to flare up again, originating from a residual focus when new susceptible individuals should appear. Amongst us

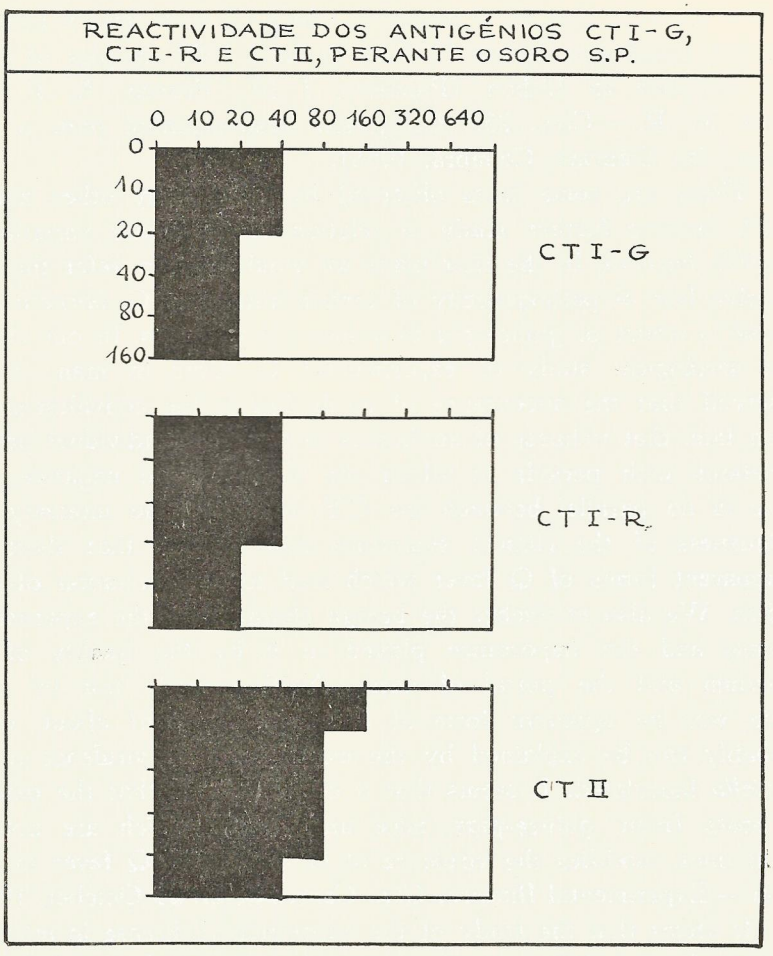

Figure 7

TABLE 1 — *Reactivity of the antigens in CF tests*

Antigens	S.P. serum (human)	C 241 serum (guinea-pig)
Phase I (Christie)	40	0
Phase II (Nine Mile) . . .	80	1 280
TC I — G	40	0
TC I — R	40	0
TC II	80	0

it was observed that in a period in which Q fever was a rarity it was not possible to isolate *C. burnetii* from 1500 samples of cow's milk supplied to Lisbon (Palmeiro, J. M., Franco, A. P., and Pinto, M. R. — Com. 23.º Congresso Luso-espanhol para o Progresso das Ciências, Coimbra, 1956).

There are some facts observed by us and by other authors which deserve further study in relation to the phase variation of *Coxiella burnetii*. In the first place we would like to refer the progressive loss of pathogenecity of certain strains of *C. burnetii* after mouse to mouse or guinea-pig to guinea-pig passages. In our clinical and serological study of experimental Q fever in man, it was observed that the appearance of antibodies in the convalescence is often late, that richness of antibodies in the same individual present variations with periods in which the C.F. can be negative, that there is no parallel between the C.F. titre and the intensity and seriousness of the clinical symptoms and, finally, that there are unapparent forms of Q fever which lead to the formation of antibodies. We also remember the benign character of the experimental disease and the importance played in it by the quality of the inoculum and the portal of entry. Neither orally nor by nasal route was an apparent form of the illness brought about, which probably can be explained by the modification of virulence of the *Coxiella* inoculated. It seems that it could be said that the passage in hosts (man, guinea-pigs, mice and eggs), which are not the usual ones, modifies the virulence of *C. burnetii* («Q fever in Portugal — Experimental Illness», *Clin. Contemp.*, III, 28 October, 1949).

It seems that the study of the experimental disease in man and in animals, in relation to phase variation can shed new light and open larger horizons.

Imprensa Portuguesa ★ Rua Formosa, 108-116 ★ Porto