COXIELLA BURNETII IN POLYMORPHIC LYMPHOCYTES IN TISSUE AND BLOOD OF PATIENTS WITH POLYMORPHIC RETICULOSIS

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Summary. – *Coxiella burnetii* is a well-known causative agent of granulomatous inflammation and an inducer of morphological changes and transformation of human B lymphocytes *in vitro*. An association of the organism with polymorphic reticulosis (PMR), a malignant granulomatous inflammation characterized by polymorphic lymphocytes, was examined. The infection of *C. burnetii* was demonstrated in all cases tested, especially in polymorphic lymphocytes. Also the presence of morphologically transformed peripheral blood lymphocytes (PBLs) infected with *C. burnetii* was demonstrated. In cultures of blood lymphocytes, *C. burnetii*-infected polymorphic cells identified as B cells became immortalized *in vitro*. These findings implicate the role of *C. burnetii* in the process of PMR.

Key words: dendritic lymphocytes; granuloma; immortalization; transformation; polymerase chain reaction

Introduction

PMR has often been referred to as lethal midline granuloma, or as a malignant facial mutilating granuloma. The disease is thought to represent a localized hypersensitivity reaction resulting in acute or chronic inflammation and granulomatous changes with polymorphic lymphocytes. However, the responsible antigen(s) remains unknown. A variety of microorganisms have been considered possible causative agents but the consistent presence of any one has not been found. *C. burnetii* is known to cause a number of diseases of granulomatous inflammation of the granuloma type (Racz and Tenner-Racz, 1980); e.g., Q fever as an acute febrile illness, chronic endocarditis, pneumonia, hepatitis, perinatal infection, and lung pseudotumor (Janigan and Marrie, 1983; Lipton *et al.*, 1987). An involvement of *C. burnetii* in polymorphic transformation of B lymphocytes was implicated in a case report of Q fever associated with hairy cell leukaemia (HCL) where a pathognomic cells were manifested as polymorphic B lymphocytes (Vuille and Delafontaine, 1989). Furthermore, we recently discovered that *C. burnetii* transformed B cells to polymorphic cells *in vitro* (Lee, 1993). The geographic range of *C. burnetii* is essentially global in wildlife and the airborne infection is the major route to human infection. *C. burnetii* is extremely infectious in human hosts. One organism is sufficient to initiate the infection (Tigertt *et al.*, 1961). Infections of *C. burnetii* were also reported in humans and animals immunosuppressed with cortisone, predenisolone, cyclophosphamide or irradiation (Sidwell *et al.*, 1964a,b; Kazár *et al.*, 1983; Heard *et al.*, 1985). In the present study, an infection of *C. burnetii* was examined in patients with PMR, especially in polymorphic lymphocytes in tissues and peripheral blood with the aim to evaluate the association of B lymphocytes with PMR.

Abbreviations: EBV = Epstein-Barr virus; FBS = foetal bovine serum; HCL = hairy cell leukaemia; IFA = immunofluorescence assay; LPS = lipopolysaccharide; MARCKS = myristoylated alanine-rich protein; MEM = minimal essential medium; MoAb = monoclonal antibody; PBLs = peripheral blood lymphocytes; PMR = polymorphic reticulosis; RFLP = restriction fragment length polymorphism; SIg = surface immunoglobulin; TEM = transmission electron microscopy; TRAP = tartarate-resistant acid phosphatase

Materials and Methods

Patients. Six cases of PMR were diagnosed on the basis of histopathological examination of biopsy tissues. The latter were

fixed in paraffin blocks (Table 1) and tested for the presence of *C. burnetii* infection by immunofluorescence (IFA) and polymerase chain reaction (PCR) assays. Morphology and cultural behavior of fresh and cultured PBLs were evaluated in relation to *C. burnetii* infection.

Table 1. Summary of the cases of PMR

Case No.	Age/Sex	Biopsy	Treatments	Symptoms
1	38/F	Nasal cavity, turbinate epiglottis, false cord	COPA, RT 9500	Nasal stuffiness, easy bleeding
2	74/M	Nasal cavity	COPA, RT 5040	Nasal stuffiness
3	53/M	Nasopharynx, oral cavity	COPA, RT 7000	
4	44/M	Tonsil	COPA –	Sore throat, swallowing difficulties
5	68/M	Nasal cavity	– RT 7000	Nasal stuffiness
6	51/F	Tonsil, tonsillar pilla	– RT 4500	Referred PMR

COPA = cyclophosphamide, vincristine, predenisolone, adriamycin.
RT = radiotherapy in cGy.
F = female; M = male.

Preparation of tissues for IFA. Paraffin-embedded tissue sections on microscopic slides were deparaffinized by heating at 60 °C for 2 hrs and soaking in xylene for 20 mins. They were then washed and fixed in cold acetone for 15 mins.

Preparation of fresh and cultured PBLs for IFA. On delivery, a drop of each blood sample was immediately prepared on microscopic slides and examined under a phase contrast microscope for morphology. For IFA, the cells were collected on glass slides by centrifugation and fixed in cold acetone for 15 mins. For cultivation, PBLs were separated from blood by Ficoll-Hypaque (Pharmacia) and seeded into plastic culture flasks (Costar) containing Eagle's Minimum Essential Medium (MEM; Hazleton Biologics) supplemented with non-essential amino acids (Hazleton Biologics), 15% foetal bovine serum (FBS), penicillin (100 IU/ /ml) and streptomycin (100 µg/ml). The cells were incubated at 37 °C in humidified CO_2 incubator. After about 5 days of cultivation, PBLs were tested for *C. burnetii* and cellular markers. They were suspended in Ca^{++}- and Mg^{++}-free phosphate buffered saline (PBS; 1 x 10^6 cells/ml), and centrifuged at 13,000 x g for 10 secs at 4 °C.

IFA. Pelleted PBLs were treated with 20 µl of the primary antibody specific to the antigen to be tested (polyclonal antibody to *C. burnetii*, monoclonal antibody (MoAb) to surface immunoglobulin (SIg); Beckton Dickinson) for 45 mins. Tissues or cells prepared were then treated with rabbit anti-*C. burnetii* serum (Pasteur Institute, France, or ATCC, USA). Cells were then washed 3 times with Ca^{++}- and Mg^{++}-free PBS and treated with fluorescein-labelled goat anti-rabbit serum (KPL, USA) for 45 mins. The cells were washed once and the tissues were placed on slides for microscopy (Zeiss Epifluorescence™, Germany). For controls, fresh normal PBLs, negative reference serum (ATCC, USA) and fluorescencein-labelled secondary antiserum were tested in various combinations.

Preparation of specimens for PCR. Target biopsy tissues or isolated PBLs were shaken with 100 µl of 0.01 mm glass beads in 50 µl of phenol : chloroform : isoamyl alcohol (25:24:1) for 1 min. The same amount of chloroform was then added and centrifuged to collect the supernatant. DNA was ethanol-precipitated from the supernatant at –20 °C. After centrifugation the pelleted DNA was washed, dried, and dissolved in 30 µl of distilled water.

PCR. A pair of oligonucleotide primers (CB1 : 5'-ACT CAA CGC ACT GGA ACC GC-3', CB2 : 5'-TAG CTG AAG CCA ATT CGC C-3') was selected according to the DNA sequence of the gene encoding superoxide dismutase of *C. burnetii*. The primers were dissolved to concentration of 0.1 mg/ml for the reaction. The length of the sequence targeted for amplification was predicted to be 257 bp. PCR was performed with a 10 µl sample in a total volume of 100 µl. The final reaction mixture contained both primers (0.1 µmol/l each), dATP, dCTP, dGTP, and dTTP (200 µmol/l each), 50 mmol/l KCl, 10 mmol/l Tris-HCl pH 8.3, 2.0 mmol/l $MgCl_2$, 0.01% gelatin, and 2.5 U of Taq DNA polymerase (Perkin-Elmer Cetus) and was overlaid with 100 µl of mineral oil. Samples were subjected to 35 cycles of amplification in a DNA thermal cycler (Perkin-Elmer Cetus). The amplification cycle consisted of denaturation at 94 °C for 1 min, annealing of primers to template at 50 °C for 1 min, and primer elongation at 72 °C for 2 mins, and final extension at 72 °C for 10 mins. PCR products were analyzed by electrophoresis in 2% agarose gel stained with ethidium bromide.

Restriction analysis. To confirm that the amplified PCR products of the expected size represent the target sequence, they were cleaved with restriction endonucleases *Alu*I and *Bgl*I known to cut within the target sequence. Samples (10 µl) of the PCR mixture were digested and then examined by 3% metaphor agarose gel (FMC; Bioproducts, Rockland, ME) electrophoresis.

Microscopy and histochemistry of PBLs. In the primary cultures, morphological transformation of the cells was examined by phase contrast microscopy (Zeiss, ICM405, Germany). To observe their morphology in detail, the cells were incubated in a microscopic chamber. The dynamic nature of cytoplasmic projections was recorded by video-microscopy. The presence of tartarate-resistant acid phosphatase (TRAP) in dendritic cells was verified the method of Yam *et al.* (1971). The presence of *C. burnetii* in cells was examined also by transmission electron microscopy (TEM; Philips CM-10) at 6,000 x to 35,000 x magnification.

Results

Pathological findings

Histopathological analysis was done on all biopsy specimens from 6 patients diagnosed as cases of PMR. Diagnostically, three parts of the tissue were considered: cellular compartment, compartment of fibrin-rich necrotic exudate, and compartment of granular tissue (Fig. 1). The cellular compartment was densely filled with polymorphic lymphocytes of small

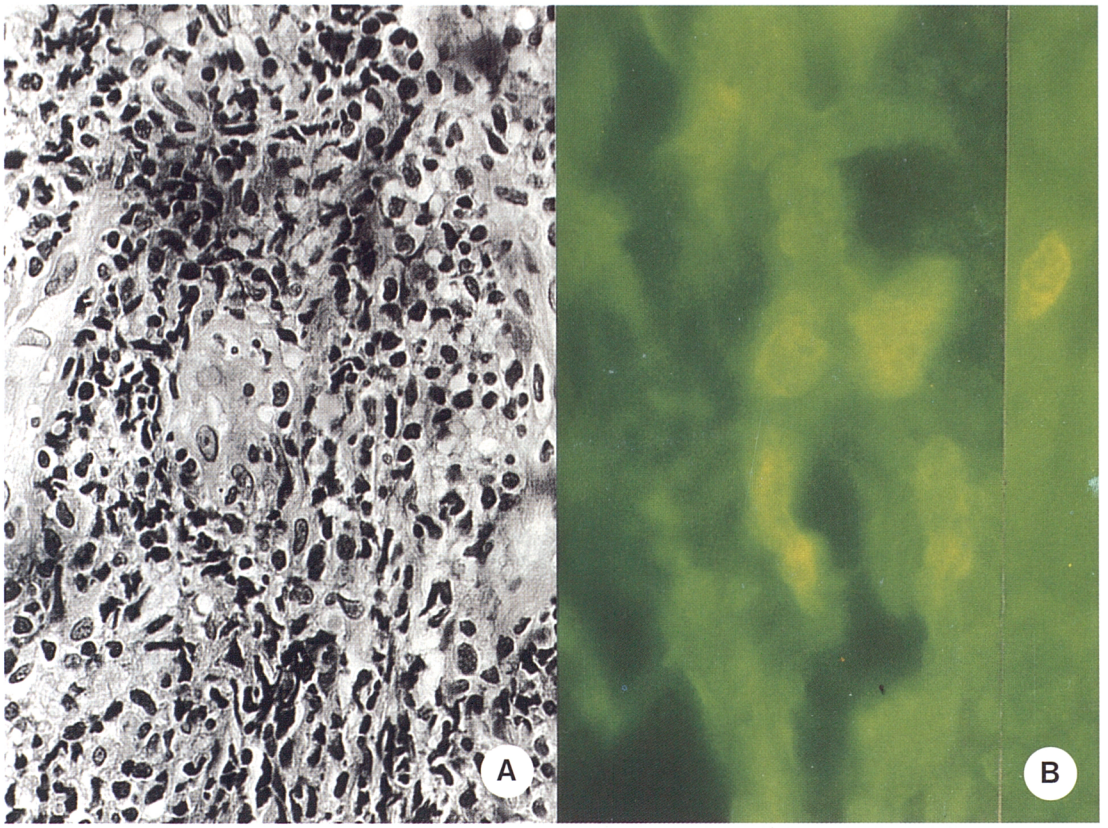

Fig. 1
Histopathological examination (A) and detection of *C. burnetii* by IFA (B) in biopsy tissue

or intermediate size. Their nuclei in scanty cytoplasm were inconspicuous. In general, they appeared to be smudged or stippled with coarse chromatin. Extreme variation in the nuclear shape was demonstrated by nuclei of oval, elongate, lobulated, baseball glove-like form or cerebriform. In some areas, lymphocytes with round nuclei in clear cytoplasm, which were proliferating in sheets, were noticed. Cells in mitosis were frequent. In the granulation compartment, proliferation of vascular walls with plump endothelial cells was obvious. The intravascular space was occupied by atypical lymphocytes similar to those seen in the cellular compartment. Activated fibroblasts and scanty amounts of interstitium were interposed. The perivascular arrangement was not conspicuous. Occasionally, destructive changes together with fibrin leakage were noticed in the vascular wall to which a few lymphocytes closely bordered. All these findings were compatible with those reported in cases of extranodal malignant lymphoma. PMR encompasses 3 stages: the early acute stage, the chronic inflammatory stage, and the stage of extranodal malignant lymphoma. Changes seen in the 1st and 2nd biopsies of the cases, especially of the case No. 1, met the histological criteria of PMR. Large anaplastic cells with hyperchromatic nuclei, plasma cells, and other inflammatory cells were admixed with the polymorphic lymphocytes.

C. burnetii detected in pathological tissues

Cells with cytoplasmic granules that specifically absorbed fluorescein-labelled anti-*C. burnetii* antibodies were detected in IFA (Fig. 1). Although the incidence of such cells was not frequent in microscopic fields, they were recognized without exception in every specimen examined. Infection of the tissue with *C. burnetii* was further confirmed by demonstrating specific gene sequences of *C. burnetii* by PCR. All the specimens positive for *C. burnetii* infection by IFA were also positive by PCR assay.

C. burnetii detected in PBLs

In immediate IFA for *C. burnetii* infection in an aliquot of fresh PBLs, all (6/6) cases were confirmed to have lymphocytes that contained IFA-positive granules in their cytoplasm. IFA-positive particles of different size and brightness were randomly scattered throughout the cytoplasm

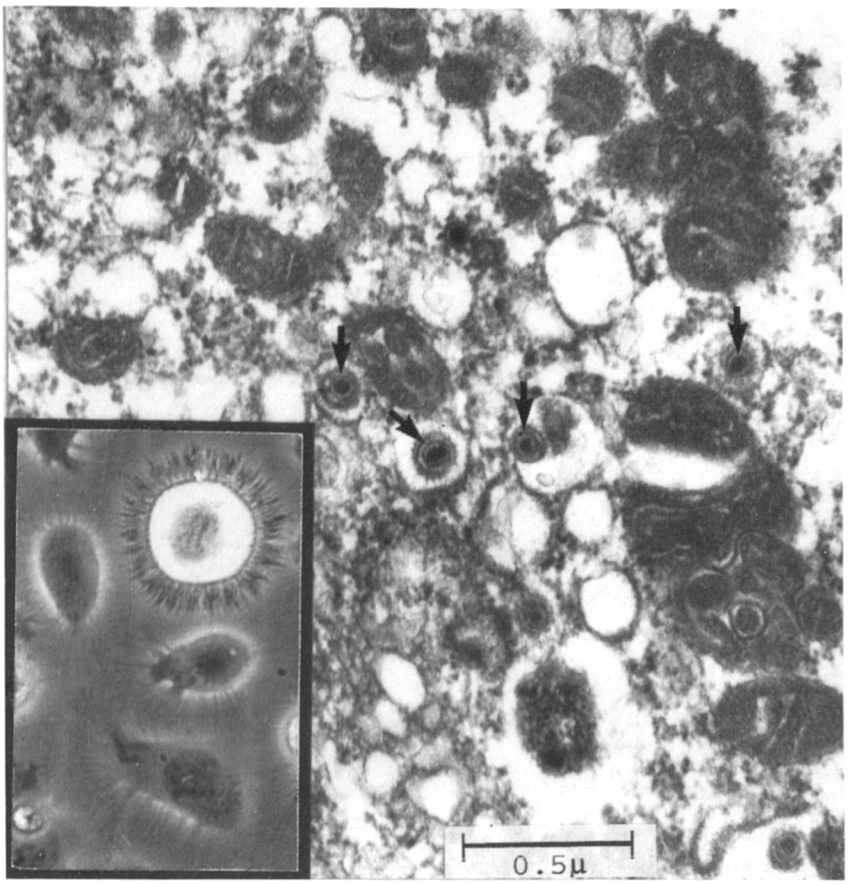

Fig. 2
Morphology of PBLs after 3 days in culture
PBLs with dendritic projections (light microscopy, insert). *C. burnetii* cells with double membrane. Spore-like electron-dense particles of diameter 0.3 μm (arrows) inside the *C. burnetii* cells are seen.

(Fig. 2a). After cultivation, the cells showed pleomorphic cytoplasm with dendritic projections budding from the cytoplasmic membrane. On the basis of ultrastructural studies of the granules by TEM, they were regarded as *C. burnetii*. The granules varied in size from 0.3 to 1 μm in diameter and had a double membrane. Spore-like electron-dense particle (0.3 μm) inside of the organism were observed. This is an unique feature of *C. burnetii* among the members of *Rickettsiae* family (Fig. 2b). By demonstrating gene sequences specific to *C. burnetii* in four cultures by PCR, the infection of *C. burnetii* in these cells was confirmed.

C. burnetii-specific DNA sequence detected in pathological tissues and PBLs

Selected samples (3 of 6 pathological tissues and 3 of 6 PBL samples) were subjected to PCR. The *C. burnetii*-specific 257 bp sequence was amplified in all of these samples. In agarose gel electrophoresis, they showed the same mobility as compared to that of the PCR product from *C. burnetii*-infected L929 cell line (Fig. 3a). In restriction fragment length polymorphism (RFLP) analysis, the PCR products were confirmed to be the targeted 257 bp DNA fragment, a part of *C. burnetii* gene for the superoxide dismutase (Fig. 3b).

Culture of C. burnetii-infected PBLs

Fresh PBLs were maintained in culture for an average of 4 months. After three days of cultivation, cells with dendritic projections dominated in all of the cultures (Fig. 2a). Newly established dendritic cell lines were found positive for both markers, TRAP and SIg, and infected with *C. burnetii* (Table 2). One of the cultures became an immortalized es-

Table 2. Detection of *C. burnetii* in pathologic tissues and PBLs of patients with PMR

Case No.	*C. burnetii* in tissues		*C.burnetii* in PBLs			Markers in cultured	
	IFA	PCR	IFA	TEM	PCR	SIg	T
1	+	+	+	+	+	+	+
2	+	+	+	+	+	+	
3	+	ND	+	ND	ND	+	
4	+	ND	+	ND	ND	+	
5	+	+	+	+	ND	+	
6	+	ND	+	+	+	+	+

ND = not done.

tablished cell line which has since been maintained for more than one year. In video-microscopy, the projections were rapidly moving in random directions. One of the cultures was immortalized as a dendritic cell line which was persistently infected with *C. burnetii in vitro*. The polymorphic PBLs were all infected with the organism and positive for SIg, a B cell marker, and secreted polyclonal antibodies *in vitro*.

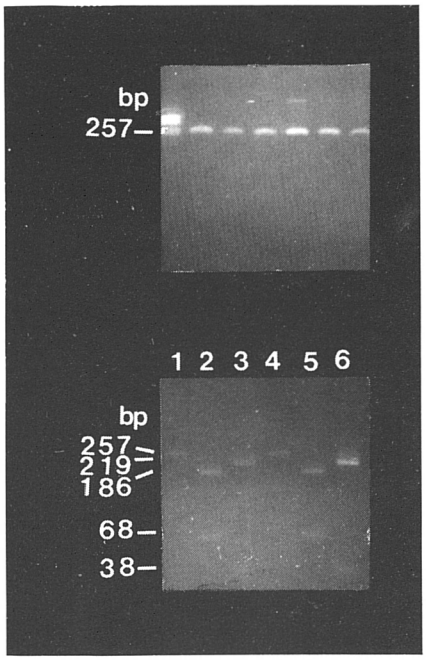

Fig. 3
Agarose gel electrophoresis of PCR products and their restriction fragments
257 bp long PCR products amplified from various samples of DNA isolated from *C. burneti*-infected L929 cell line (upper part, lines 1-5). Undigested (lanes 1, 4), and *AluI* (lanes 2, 5) and *BglI* (lanes 3, 6) digested 257 bp PCR products amplified from *C. burnetii*-infected L929 cell line (lower part).

Discussion

Polymorphic reticulosis is thought to represent a localized acute or chronic inflammation resulting in granulomatous changes with polymorphic lymphocytes. However, the responsible antigen(s) remain(s) unknown. In this study we observed that all patients with PMR were infected with *C. burnetii*. The role of this organism in polymorphic transformation of lymphocytes was demonstrated by detection of *C. burnetii* not only in the characteristic lymphocytes of small or intermediate size in the granulomatous tissues but also in the polymorphic lymphocytes detected in the peripheral blood.The polymorphic PBLs were cultivated *in vitro* and these were found positive for SIg and secreted polyclonal immunoglobulins *in vitro*. Cytoplasmic projections of the polymorphic PBLs were found to be motile and metamorphic in video-microscopy. An intracellular movement of *C. burnetii* may be responsible for this since periodic contractions and extrusion of the organism-bearing cytoplasmic membranes in the polymorphic PBLs was also noticed. These findings correspond to our previous ones showing that *C. burnetii* caused polymorphic transformation of B lymphocytes *in vitro* and that polymorphic B cells infected with *C. burnetii* could be demonstrated in *Coxiella*-positive blood samples (Lee, 1993).

Although the role of *C. burnetii* in transformation of B cells is not clear, it is known that certain bacteria, e.g. *Shigella* spp. (Maurelli *et al.*, 1992) and *Listeria monocytogenes* (Tilney and Portnoy, 1989), induce changes in cell shape (e.g. during formation of pseudopods for movement or engulfment) by rearrangement of actin filaments. In those reports, two types of intracellular movement of bacteria in cytoplasm, namely the intercellular spread and the organelle-like movement, are described in infections with *Shigella* spp. and *L. monocytogenes*. Furthermore, also *C. burnetii*-induced morphological changes in human and animal lymphocytes were reported. These include extrusion of the organism-bearing membranes of *C. burnetii*-harboring cells (Khavkin and Amosenkova, 1981) and emergence of "limbocytes" in humans and animals (Pough and McPherson, 1985; Hume *et al.*, 1983), and "podocytes" in a monkey kidney cell line after *C. burnetii* infection (Burton *et al.*, 1978). Purified bacterial lipopolysaccharide (LPS) was reported to induce development of filamentous arrays in human B cells (Albrecht *et al.*, 1990) and "spike-like structures" in macrophages (Shinji *et al.*, 1991). Thus, the LPS of the organism is considered responsible for the morphological changes in *C. burnetii*-infected B cells. Recently, the molecular biology of LPS-induced cytoskeletal modulations in a macrophage has been disclosed. LPS-induced myristoylated alanine-rich protein acts as a substrate for protein

kinase C (MARCKS; Rosen *et al.*, 1990). Activation of protein kinase C resulted in displacement of MARCKS which then binds actin filaments and causes the filopodia formation in macrophages.

In addition to the morphological transformation, the immortalization of polymorphic PBLs was demonstrated *in vitro*. These cells proliferated for more than 6 months *in vitro*. One of these cultures became an established cell line of polymorphic lymphocytes which proliferated for more than 1 year *in vitro*. Although *C. burnetii* in B cells was reported not to harm their proliferation (Khavkin, 1990), *C. burnetii*-induced immortalization of mammalian cells, especially human B cells, has never been reported. Epstein-Barr virus (EBV) is the only known agent to immortalize human B cells *in vitro*. The role of EBV in our experiments cannot be excluded, since the polymorphic cells were all positive for EBV antigens (EBNA1,2; data not shown). Thus we assume that the role of *C. burnetii* in the immortalization of B cells may be an activation of latent EBV infection. The polymorphic PBLs were positive for *C. burnetii* and SIg, and secreted polyclonal antibodies *in vitro*. For tissue lymphocytes, a marker study was not available, however, their B cell nature was probable since the B cell tropism of *C. burnetii*, especially for the persistent infection, was reported from animal experimentation (Khavkin and Tabibzadeh, 1988).

Nevertheless, *C. burnetii* itself causes acute or chronic diseases, especially in immunosuppressed patients. Thus, the testing for *C. burnetii* infection in PMR is recommended, especially in endemic regions. Fortunately, *C. burnetii* is susceptible to antibiotics and appropriate vaccines are available. The response of the patients with PMR to antibiotics recommended for *C. burnetii* infection is under study.

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