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REVIEW ARTICLE

Q fever – selected issues

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Abstract

Q fever is an infectious disease of humans and animals caused by Gram-negative coccobacillus *Coxiella burnetii*, belonging to the *Legionellales* order, *Coxiellaceae* family. The presented study compares selected features of the bacteria genome, including chromosome and plasmids QpH1, QpRS, QpDG and QpDV. The pathomechanism of infection – starting from internalization of the bacteria to its release from infected cell are thoroughly described. The drugs of choice for the treatment of acute Q fever are tetracyclines, macrolides and quinolones. Some other antimicrobials are also active against *C. burnetii*, namely, telitromycines and tigecyclines (glicylcycline). Q-VAX vaccine induces strong and long-term immunity in humans. Coxevac vaccine for goat and sheep can reduce the number of infections and abortions, as well as decrease the environmental transmission of the pathogen. Using the microarrays technique, about 50 proteins has been identified which could be used in the future for the production of vaccine against Q fever. The routine method of *C. burnetii* culture is proliferation within cell lines; however, an artificial culture medium has recently been developed. The growth of bacteria in a reduced oxygen (2.5%) atmosphere was obtained after just 6 days. In serology, using the IF method as positive titers, the IgM antibody level >1:64 and IgG antibody level >1:256 (against II phase antigens) has been considered. In molecular diagnostics of *C. burnetii* infection, the most frequently used method is PCR and its modifications; namely, nested PCR and real time PCR which detect target sequences, such as *htpAB* and *IS1111,* chromosome genes (*com1*), genes specific for different types of plasmids and transposase genes. Although Q fever was diagnosed in Poland in 1956, the data about the occurrence of the disease are incomplete. Comprehensive studies on the current status of Q fever in Poland, with special focus on pathogen reservoirs and vectors, the sources of infection and molecular characteristics of bacteria should be conducted.

Key words

Coxiella burnetii, intracellular parasite, *htpAB, IS1111* genes, bioterrorism, epidemiology

INTRODUCTION

Q fever (*latin: febris Q, coxiellosis)* occurs worldwide among many animal species and, secondary, in humans. It is caused by a taxonomical and phylogenetically unique bacteria (*C. burnetii*), proliferating intracellulary and producing spores which are exceptionally resistant to physico-chemical factors. The WHO/FAO/OIE and EFSA/ECDC include it among 'emerging infectious diseases' [1, 2].

Q fever was described in 1935 in Australia [3] in 9 employees of a slaughterhouse in Brisbane, capital of the state of Queensland. The outbreak of acute disease with fever and influenza-like symptoms was observed and described by Derrick, who named it 'Query' (Q) fever [4]. He inoculated guinea pigs with the blood and urine of patients, which caused the illness to develop in the animals. Because Derrick was unable to detect any bacteria in the infected guinea pig tissues, he sent a saline emulsion of infected guinea pig's liver to Frank MacFarlane Burnet (the future Nobel laureate). Burnet and Freeman [5] examined Giemsa and Castaneda-stained specimens of mice spleens infected with this material, and found 'bodies which appear to be of rickettsial nature, sometimes in enormous numbers'.

In 1939, the identity of Q fever and 'Nine Mile fever', a disease occurring in the Nine Mile Creek region of Montana, USA, was demonstrated. In 1939, Cox isolated the bacteria from infected chicken embryos. In honour of the above-mentioned discoveries the bacterium was named *C. burnetii* [6, 7, 8, 9]. The acute fever disease with atypical pneumonia occurred during World War II among hundreds of German soldiers in Yugoslavia, Greece, Bulgaria ('Balkangrippe'), Italy and Corsica, as well as in the Ukraine, including Crimea. After the end of war, in cooperation with American and German bacteriologists, the ailment was recognized as Q fever [10].

Q fever also appeared among American and British soldiers during the landings in Sicily and Italy in the winter of 1944 and spring of 1945 [11]. At that time, the Henzerling strain isolated from the blood of an American soldier, together with the Nine Mile strain, have become the standard strains commonly used today. More recently, in 1990, Q fever appeared among the American military staff during the Gulf War [11].

The infection of humans occurs most often through direct contact with infected animals, e.g. in slaughterhouses, tanneries, fur, meat, leather and wool processing industries, and employees of agricultural farms, veterinarians, etc. [12, 13, 14, 15, 16]. Infection may be aquired via the aerosol route, ingestion (contaminated raw milk), direct skin or mucosa penetration, and as a result of tick bites. Q fever is manifested with bacteriemia and disease symptoms. There are various forms of the clinical symptoms of Q fever in humans, among them, acute and chronic types (generally associated with endocarditis*)* can be distinguished.

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Etiological agent. *C. burnetii* is small, short and pleomorphic gram-negative coccobacillus with approximate dimensions of 0.2 by 1.0 μm. For many years it has been classified in the order *Rickettsiales*, despite many differences from others rickettsiae. In recent years, the taxonomic reclassification of the microorganism has been carried out. *C. burnetii* has been removed from the group rickettsiae. In addition, molecular phylogenetic studies (mainly using 16S rRNA sequencing) showed that in contrast to rickettsiae derived from *Alphaproteobacteria*, *C. burnetii* originates from quite different ancestors – from *Gammaproteobacteria*. Finally, it has been established that *Coxiella* shows the nearest, although still quite distant relationship with *Legionella*, the bacteria from the *Legionellales* order, which enabled the formation of 2 families: *Legionellaceae* and *Coxiellaceae*, with genus *Coxiella*, and one species *C. burnetii* [17]. *C. burnetii* exists in 2 morphologically distinct forms: LCV (Large Cell Variant) and SCV (Small Cell Variant) [18, 19, 20, 21, 22].

Small Cell Variant is characterized by exceptional resistance to physical (e.g. ultraviolet radiation) and chemical factors. In dried milk the bacterium is able to live up to 30 days, in urine 49, in the dust about 120 days, while in the *Dermacentor andersoni* faeces for up to 580 days. In wool stored at 4–6°C, the bacterium can survive for even 12–16 months, and in soil up to 5 months [16]. The temperature of 61.7°C can inactivate bacterium in raw milk within 20 minutes, while in the liquid suspensions the inactivation takes after one hour at 80°C. Gamma rays can also be used to inactivate the pathogen (6.6×10^5 rads destroy bacteria in 90%) [11]. In dry, dusty, tick faeces (e.g. on the surface of animal skin or hair) bacteria may survive for even 6 years, and penetrate the human or animal organism through the respiratory tract or conjunctivae causing infections. The media inactivating bacteria are: 2% hot solution of sodium hydroxide (NaOH), 5% phenol solution, 10% chlorinated lime solution, 5% chloroform and 70% ethyl alcohol, 2% formaldehyde and 5% hydrogen peroxide [6, 7, 9, 23].

***C. burnetii* genome**. The genome of *C. burnetii* Nine Mile phase I consists of 1.9 Mb chromosome and plasmid QpH1 (37,393 bp) [24]. In the National Centre for Biotechnology Information (NCBI) database, there are 6 available genomes of *C. burnetii*, among which 4 are fully sequenced and 2 are still being analyzed. The comparison of 4 sequenced genomes of different *C. burnetii* strains is shown in Table 1.

Table 1. Comparison of *C. burnetii* genomes [25, modif.].

Properties	*C. burnetii* strain			
	Nine Mile	K	G	Dugway
Size of chromosome (bp)	1,995,281	2,063,100	2,008,870	2,158,758
Coding regions (%)	90.7	90.3	89.7	90.7
GC content (%)	42.7	42.7	42.6	42.4
Number of ORFs				
Total ORFs	2,227	2,325	2,300	2,265
With known function	1,348	1,441	1,403	1,391
With unknown function	879	884	897	874
Pseudogenes (total pseudogenes)	413 (197)	476 (244)	484 (224)	265 (136)
Transposases (pseudogenes)	31 (1)	59 (27)	40 (7)	32 (20)

C. burnetii Nine Mile phase I, *C. burnetii* strain K, *C. burnetii* strain G, *C. burnetii* strain Dugway 5J108111.

All strains of *C. burnetii* possess 1 autonomous plasmid with size ranging from 37–55 kb, and in some cases plasmid sequences can be integrated with bacterial chromosome [25]. High homology among plasmid sequences has led to the hypothesis that they are essential for the survival of the pathogen [26]. This hypothesis was confirmed with the PCR-RFLP (Polymerase Chain Reaction – Restriction Fragment Lenght Polymorphism) method, and as a result, 6 different genomic groups (genomic groups I-VI) have been characterized [25]. To ensure that the PCR-RFLP is a sufficient method, MLVA (Multilocus Varaible Number Tandem Repeats Analysis) analysis [27] and the MST method [28] were also performed. The results suggest that all of the above phylogenetic methods can be successfully used in molecular analysis of *C. burnetii*. A novel approach in molecular testing was application of the microarray method in the molecular characterization of isolated strains. This method revealed 2 new genomic groups of *C. burnetii* (VII and VIII) [29]. The molecular characteristics of *C. burnetii* strains also included sequencing and the PCR-RFLP of specific genes: *icd* (isocytrate dehydrogenase) [30], *com1* (outer membrane protein) and *mucZ* (mucoid protein) [31].

Plasmids. The *C. burnetii* genome may contain one of 3 different plasmids: QpH1m QpRS and QpDG. QpH1 and QpRS are autonomic structures, while in G isolate, the plasmid sequence is integrated with the chromosome [25]. Comparison of some properties of plasmids are shown in Table 2. The QpRS plasmid (39,280 bp) of Priscilla Q177 *C. burnetii* strain, possess 17 polymorphic sites, influencing the 8 ORF's (Open Reading Frames). Strain Dugway carries QpDG plasmid (54,179 bp) [25]. Plasmid sequences of isolate G are integrated with chromosome between two ORFs: CbuG007 and CbuG0090, encoding hypothetical proteins [32]. On the nucleotide level, QpH1, QpRS, QpDG and plasmid-like sequences of isolate G show 99% homology in common 14,218 bp fragments. QpH1, QpRS and QpDG have common 28,421 bp fragments which show 99 % homology. Additionally, QpH1, QpRS and QpDG possess highly specific sequences, 3,685 bp, 2,677 bp, and 15,243 bp, respectively. QpRS and QpDG share a common 34,940 bp sequence [25].

Table 2. Comparison of *C. burnetii* plasmids [25].

Properties	Plazmid		
	QpH1	QpRS	QpDG
Size of plasmid (bp)	37,393	39,280	54,179
Coding regions (%)	81	79.6	84.9
GC content (%)	39.3	39.7	39.8
Number of ORFs			
Total ORFs (inc. pseudogenes)	50	48	66
Known function	19	20	26
Unknown function	32	28	40
Total number of pseudogenes	15 (10)	10 (6)	13 (7)
Transposases	0	0	1

Types of plasmids were related to the previously characterised genomic groups [29, 33, 34].

C. burnetii strains isolated from humans with 'acute' symptoms, in most cases belong to I, II and III genomic group, while plasmids from IV and V genomic group were

found in patients with 'chronic' symptoms. This correlation led to the hypothesis that plasmids encode specific virulence factors which can determine the different virulence levels of *C. burnetii* strains [29].

C. burnetii isolates with QpH1 plasmid are correlated with 'acute' symptoms, while isolates with QpRS plasmid are responsible for 'chronic' symptoms. Another plasmid, QpDG is known to be non-pathogenic for humans [24]. Genomic group I, II and III is mainly represented by QpH1 plasmid, group IV by QpRS plasmid (strain Q177 Priscilla, K Q154 and P Q173). Group V (Corazon) includes strains with integrated plasmid-like sequences with chromosome (strains G Q212, S Q217 and Ko Q229). This group contains the *cbbE* gene encoding of a specific 55 kDa E protein of the cell wall. The Corazon strain is probably responsible for the development of human endocarditis [35].

Group VI contains only isolates with QpDG plasmid which was found in Dugway 7E9–12 strain of *C. burnetii*. QpDV plasmid was not compared with any of the above-mentioned genomic groups [29]. Genotypic studies revealed the existence of at least 34 *C. burnetii* sequential types, which may also be co-responsible for the acute or chronic forms of the disease [28].

Pathomechanism of *C. burnetii* infections. Infection by *C. burnetii* is most commonly caused by inhalation of infectious aerosols. When the bacteria penetrate into the host's organism they are phagocytized mainly by macrophages, where a process of pathogen replication takes place in the phagolysosomes. This results in phagocyte's destruction and spread of the infection in the organism. In the life cycle of *C. burnetii* the bacteria exist in 2 anti-genically different phases (variants), since the changes in the surface antigens occur during the transition between the phase I (virulent) and phase II (avirulent). In the natural environment, *C. burnetii* exists in the phase I, while in *in vitro* conditions, such as tissue culture, the bacteria loose the characteristic surface antigens and transform into the less virulent phase II. This transition is reversible, and in the experimentally-infected animals the phase II strains transformed back into the phase I bacteria [11]. Genetic studies have revealed that there is a relationship between the pathogenecity of the *C. burnetii* strains and the presence of different plasmids. This allowed classification of the pathogen into several genetic groups: I, II, III, IV, V, VI [29]. *In vitro* serial passages of phase I *C. burnetii* in chicken embryo yolk sacs or tissue cultures induce formation of bacteria producing LPS of lower molecular mass, described as phase II of this microorganism. However, both phases (I and II) have similar kinetics of replication which takes place in phenotypically indistinguishable lysosome-like PV (parasitophorous vacuoles) structures [36, 37, 38].

LPS is the major component of the outer membrane of this Gram-negative bacteria, which makes it an important factor in the virulence of *C. burnetii*. Lipid residues of phase I and II LPS have an identical chemical structure and are able to block the TLR-4 receptor in the course of the host cell's response to infection. Phase I microorganisms interact with THP-1 leukocyte receptor: $\alpha_v\beta_3$ integrin, whereas the phase II *C. burnetii* additionally binds with CR3 receptor ($\alpha_M\beta_2$ integrin) of macrophages. The phase I pathogen has a complete structure of LPS, which restricts the binding of bacteria with CR3. In contrast, the LPS of phase II *C. burnetii* has hydrophobic properties due to a lower content of carbohydrate residues, which leads to more intense phagocytosis of phase II bacteria by host phagocytic cells [37, 38].

C. burnetii exists in 2, morphologically distinct forms: vegetative – known as LCV (large cell variant), which shows a less dense structure under transmission electron microscopy, and a spore-like form – SCV having a more dense structure [7, 14, 39]. SCVs (0.2–0.5 µm in size) is metabolically-inactive, and is able to survive in the environment outside the host for a very long time due to its resistance to various physical factors, such as high temperature, or disinfection agents. The larger, 1.0 µm LCV form, has high metabolic activity and lower resistance to environmental conditions [6, 8, 9]. Conversion of SCV into LCV takes place in parasitophorous vacuoles (PV) rich in amino acids, carbohydrates and peptides regulating bacterial metabolism. *C. burnetii* replicates in phagolysosomes, and is the only known bacterial pathogen whose multiplication during the life cycle occurs in the PVs. According to many authors, this characteristic feature constitutes the most important element of the pathomechanism of *C. burnetii* infection [36, 37, 38].

Internalization of *C. burnetii* into host cells occurs via microfilaments-dependent endocytosis. Adherence of the *C. burnetii* virulent phase I to human monocyte-like THP-1 cells leads to protrusion of the host cell membrane to the site of bacteria adhesion. Phase I of the microorganism interacts with the $\alpha_v\beta_3$ integrin receptor on THP-I leukocytes, whereas the phase II is additionally able to interact with CR3 [37].

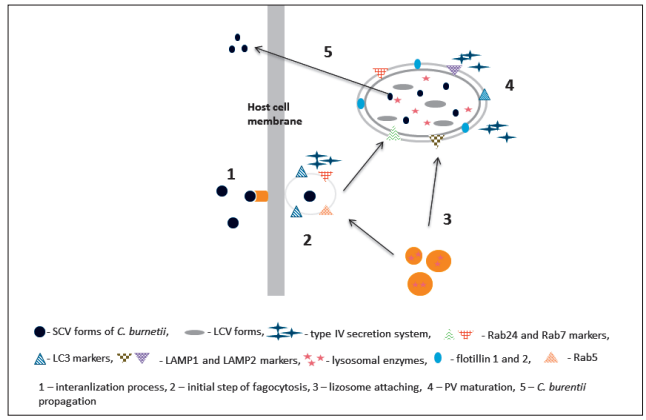

Figure 1. Infection scheme of *C. burnetii* (author's schedule).

During the first 6 hours after internalization, an interaction of *C. burnetii* with the host's autophagosomes and endosomes takes place, manifested by the presence of autophagic marker LC3 (microtubule associated protein – Light Chain 3) and endosomal proteins: Rab5, Rab7. This process requires the presence of effector proteins in a form homological to the Dot/Icm type IV secretion system (T4SS) of *Legionella*, which indicates the close phylogenetic relationship between the 2 microorganisms. The common feature of the bacterial secretion effectors is the presence of eukaryotic-like motives that functionally mimic the activity of the host cells' proteins. Additionally, at this time, Akt and Erk1/2 kinases become activated, inhibiting the apoptotic programmed cell death [37].

In the next stage of PV formation (around the second day post-infection), the phagosome containing *C. burnetii* fuses with lysosome, which leads to activation of lysosomal enzymes: acidic phosphatase and cathepsin D. The transformation of the resistant, metabolically inactive SCV into the LCV form, which actively replicates and shows high metabolism,

takes place in the maturing parasitophorous vacuoles, similar to other species of bacteria from *Legionella spp.* and to protozoans from *Leishmania spp.* [37, 40]. In normal conditions, autophagy is a process used by cell to degrade cytoplasmic material and remove damaged organelles through their transport into autophagolysosomes. *C. burnetii* adapted this mechanism as a source of nutritional compounds necessary throughout the life cycle of this microorganism, and as a first step of PV membrane formation. Multiple fusion with autophagosomes containing endolysosomal vacuoles may have a fundamental role in providing the space in vacuolar membrane needed for the enlargement of PVs [37, 39, 40, 41]. At this stage of PV formation, the vacuoles are characterized by a moderately acidic environment (pH=5.0), contain acidic hydrolases (i.e. acidic phosphatase, 5'nucleotidase and cathepsin D), have surface markers: LC3, Rab7 and Rab24, as well as H^+ ATPase, and obtain additional protein markers connected with lysosomal membrane, i.e. lysosomal glycoproteins: LAM 1, 2, 3, and flotillin 1, 2 [37, 40]. Additionally, the PV membrane is enriched with cholesterol, which plays an important structural role and mediates signal transduction during *C. burnetii* infection. In terms of the structural function of cholesterol, it enhances the mechanical resistance of the double lipid layer; thus, the mature PV containing bacteria becomes highly resistant to mechanical damage, despite the large size of the vacuoles. Cholesterol also decreases the membrane permeability to ions, which is helpful in sustaining the acidic pH of the PV, as it minimizes the protons' escape. Cholesterol, together with lipoproteins: flotillin-1 and -2 is involved in signal transduction, and modulation of many cellular functions, such as membrane fusion. PV is constantly binding with the fluid phase of endosomes, which is why the endosomal fluid-phase markers are constantly present in the lumen of the vacuoles. The *C. burnetii* SCV undergoes morphological transformation into LCV, which can then start the process of replication.

On the second day of infection, a decrease of caspase activity is observed in the host cells, connected with induction of the pro-survival transcriptional response. This includes activation of Akt and Erk1/2 kinases, as well as reduction in cytochrome c release from the mitochondria [37]. These processes protect *C. burnetii* from apoptotic cell death mechanisms. Additionally, the microorganism controls apoptosis by regulation of autophagy, since it modulates the interplay between Beclin 1 (one of the major proteins of the complex in charge of autophagic nucleation in mammals) and anti-apoptotic protein Bcl-2 [42].

C. burnetii, similar to many other Gram-negative bacteria, activates the type 4 secretion system (T4SS) to modulate specific cell processes of the host, making them beneficial for bacteria. The effector proteins synthesized and released regulate the vesicular transport enabling the formation of replicative vacuoles [37, 40]. The common feature of bacterial secretion effectors is the presence of eukaryotic-like motifs, which imitate the functional activity of host cells' proteins. New effector particles include families of proteins having coiled-coil domains (CCDs), and domains with tetratricopeptide repeats (TPRs), leucine rich repeats (LRR) and ankyrin repeats (Anks). All of these structural motifs participate in various types of protein-protein interactions and cell signaling pathways [37].

The late form of PVs (observed after 6 days post-infection) constantly regulate the process of autophagy and endosomal pathway. PVs are filled with *C. burnetii* bacteria being in the stationary growth phase. The replicative vacuole grows to the stage in which it finally occupies almost the entire cytoplasm of the host cell, leading to transformation of LCV into SCV. SCV multiplies, producing morphologically new forms of *C. burnetii,* which after the release from the infected cells are able to spread the infection further to new host cells [37, 40, 41].

Epizootiology and epidemiology. At least 96 mammal species and marsupials constitute the reservoir of *C. burnetii*. Among breeding, domestic and laboratory animals, there are mainly horned cattle, sheep, goats, cats, dogs, guinea pigs and gerbils. The infection has been also detected in annelids, fish, reptiles (e.g. vipers and snakes) and birds [19, 43, 44, 45]. In nature, free living animals such as small and insectivorous rodents, bats (*Nyctalus leisleri* in Poland), shrew, wild boar, hamsters, rabbits, deer, morningstar hares, bison and bears are considered the reservoir of infection [44]. The source, and at the same time, the vector of this bacteria, are also blood-sucking arthropods (mainly ticks, Trombiculidae and other mites, mosquitoes, lice and fleas) [19, 23, 43]. Other arthropods (flies, cockroaches, lice) can be a passive vector of the disease.

In animals, *C. burnetii* is characterised by a high affinity to lung cells, mammary glands, testicles, lymph nodes (especially knots 'above the udder'), uterus and foetal fluids. It is excreted with milk, urine and faeces, as well as the amniotic fluids and placenta. In breeding animals, the infection may be asymptomatic and usually lasts the entire period of their lifetime. Intensive proliferation of bacteria and intensification of signs of the disease can occur during pregnancy or as a consequence of treatment with immunosuppressant agents. The immune status of the animals and bacterial virulence affect the development of the disease, which may present symptoms of pneumonia, inflammation of the spinal cord, eyes, and udder, as well as arthritis. The observed cases of people with symptoms of Q fever indicate the presence of this disease among animals [8, 11].

In order to explain the role of ticks in the epidemilogy of Q fever in Poland, Wierzbanowska *et al.* [46], between 1993–1995, tested 1,580 ticks collected from humans, animals and plants, using the PCR method. The presence of sequences characteristic for *C. burnetii* was found in 3 samples (0,19%). Recent findings carried out by Niemczuk *et al.* [47], who used real time PCR in their studies, have shown that the percentage of positive results in samples isolated from ticks collected from places where local outbreaks occurred, accounted for 33.3%, which may suggest that ticks can be an important vector for *C. burnetii*. In samples taken from ticks, the concentration of bacterial cells varied from 10^8–10^{11} per tick [48]. Ticks that are the natural reservoir of *C. burnetii* constitute an important element in the circulation of this microorganism in the natural environment. This agent can be carried by over 40 species of ticks which are capable of transovarial transfer of the pathogen to the next generation. It is important to note that during their life cycle, ticks can be found in 2 or more hosts, which is why these arthropods play a crucial role in the environmental persistence of *C. burnetii*. Ticks become infected during the consumption of blood with *C. burnetii* cells, and as a result, they become a carrier of the above-mentioned microbes [9, 14, 15, 49]. Among the tick species found on the territory of Poland and Slovakia,

the most significant epidemiological and epizootiological role in Q fever transmission seems to be played by *Ixodes ricinus,* but other species, e.g. *Dermacentor reticulatus*, *D. marginatus* and *Rhipicephalus sanguineus* must also be taken into account [19, 46, 50, 51].

High infectivity of *C. burnetii* (one bacteria cell can cause infection and symptoms appearance) is the main factor conducive to the spread of the disease [6, 9, 14, 52, 53]. Resistance of the bacteria against extremely harsh environmental conditions (physical and chemical factors) allows the spreading of the pathogen through air over long distances (many kilometers from the source of infection), which has a significant influence on Q fever territory range. Properties of the pathogen, especially its high virulence, causes a strict international surveillance of all research activities linked to *C. burnetii*. Risk of *C. burnetii* infection for laboratory personnel was qualified as high (classification to the risk group 3) according to the EU Directive 2000/54. All scientific and diagnostic tests involving this bacteria must be held in BSL3 laboratories [6, 7, 9, 11, 39, 49].

Q fever outbreaks have been registered on all continents, except New Zeland [54]. The dynamic progress in tourism, higher animal production, increased international trade in animals and products of their origin, as well as intensive travel by people between countries, are the main factors contributing to the higher risk of 'importing' and spreading Q fever worldwide [55, 56]. In Poland between 1993–2009, 9 cases of imported Q fever in humans was described [57].

Q fever in Poland. In Poland, the first Q fever outbreak was identified in 1956 in Owczary, Nowy Sącz Province in southern Poland. The source of infection was sheep imported from Romania [58]. Sixty-three people fell ill, and one child died. In 1957, a laboratory infection occurred, resulting in 17 persons becoming ill. Between 1959–1982, no indigenous infections were detected in humans. In 1982, however, a Q fever outbreak was reported in a herd of 220 dairy cows in Ulhówek, Lublin Voivodeship, in southeast Poland near the Ukraine border, which also resulted in the infection of at least 1,300 people [35, 59, 60, 61]. This was the largest outbreak in humans in the world – similar scale of the disease was only noted during the epidemic between 1947–1948 in Tübingen, Germany – until 2007, when a large human and animal Q fever outbreak occurred in The Netherlands. The source of disease has never been established. In 1962, 1985 and 1982–1986, imported leather goods were the cause of 3 outbreaks in humans [12, 13, 23, 35], after which, between 1986–2004, only isolated cases of the disease were reported in Poland [13, 35, 62].

In 2003, Cisak e*t al.* [63], using serological methods, examined farmers from the Lublin Voivodeship who had suffered in the past from ailments of the respiratory system. Among 90 tested sera, 16 (17.8%) samples were positive for phase I antigens, and positive titres were more frequent in females than in males. Based on these results, the authors concluded that Q fever outbreaks had probably occurred several years earlier [63].

According to the annual reports by the Chief Sanitary Inspectorate (CSI), in 2005, 59 cases of Q fever were registered (53 cases in the Lesser Poland Voivodeship and 6 cases in the Silesia). The source of infection were animals (cattle, sheep, goats) imported from The Netherlands. In recent years, Q fever outbreaks have been registered in the so-called Polish 'eastern wall'. Interdisciplinary clinical, genetic and seroepidemiologic studies have been undertaken to investigate these outbreaks [35, 47, 51, 57]. In 2008, 24 cases of human infection were registered, including 21 in Subcarphatian Voivodeship and 3 cases in Lublin Voivodeship. In 2009, 5 more persons became ill in the above-mentioned regions. The outbreak concerned workers of 2 large households, and their family members who consumed raw milk from infected cows. An exchange of cattle and travelling of employees occurred between these farms. In both Poland and throughout Europe, there are in fact many more cases of the disease in humans which remain unrecognized – the 'underestimation' phenomenon. Between 1956–2009, 258 human cases were registered in Poland, while between 2010–2011, no cases of infection were reported (Tab. 3).

However, according to epidemiological estimation of the authors of the presented study [35], Q fever in humans is diagnosed in minimal percentage of cases in Poland – only 1%. Thus, it is an 'iceberg phenomenon' of a very disadvantageous rate of recognized cases in relation to the actual number of infections. The diagnosis of Q fever makes it very difficult, it is often confused with other diseases, such as seasonal flu, and comprehensive studies are performed only sporadically [23, 35]. Close contact of people with farm animals poses an important epidemiological risk. The great Q fever epidemic (up to 4,000 human cases until 2012) in the The Netherlands, ongoing since 2007 [64], proves the legitimacy of this hypothesis.

Some authors suggest the possibility of a cyclic occurrence of the disease at 5–10 year intervals [44]. The occurrence of outbreaks of the disease in humans is illustrated in Table 3, and in animals in Table 4. According to the EFSA Report, in Poland in 2007, among 91 serologically-tested samples from cattle, 2.2% were positive; while in 2008 year, among 1,130 tested cattle, 40.1% were positive: 'The majority of samples were investigated due to clinical suspicion or after abortion, and were examined using serological tests' [2]. Based on the Regulation of the Minister of Agriculture and Rural Development of 24 June 2010, monitoring tests for Q fever are conducted in Poland. If the test result for antibodies to *C. burnetii* is positive, the sample is sent to the National Reference Laboratory (NRL) for re-implementation of the study. The samples confirmed as positive are tested using real-time PCR. The positive results in the presented study allows the unambiguous confirmation of the cases of Q fever. The results regarding the prevalence of Q fever in animals are presented in Table 4, and indicate that the disease is detected in animals throughout the country. In 1989, the circulation of the agent in nature in the form of naturally occurring infection focuses was demonstrated.

In 2009 and 2010, serological analyses of 120 samples from cattle suspected of *C. burnetii* infection was performed, showing 21.6% positive results. Six blood samples from humans having contact with the animals, in which the presence of antibodies against phase II were detected, was also examined. Genetic tests with the use of the real-time PCR method produced positive results in all analyzed human blood samples [47].

Chmielewski *et al.* [65] conducted a phylogenetic analysis of 6 *C. burnetii* strains (755, Hum, Zam, 801, 507 and Dowg) acquired from humans and animals using (Multi Spacer Typing (MST) and MLVA methods. The origin of the strains was as follows: 755 – Gorlitz, Hum – Warsaw, Zam – Zamość,

Table 3. Reported cases of Q fever in humans 1956–2011 (according to Knap 2009 [35], revisited by Knap 2012).

Year	No. of cases	Incidence per 100,000
1956	63	0.180
1957	21	0.060
1958	17	0.048
1959–1962	-	-
1962	26	0.072
1963–1979	-	-
1980- 1983*	-	-
1984	3	0.008
1985	1	0.003
1986	2	0.005
1987	2	0.005
1988	2	0.005
1989	-	-
1990	2	0.005
1991	2	0.005
1992**	20	0.052
1993	1	0.003
1994	-	-
1995	1	0.003
1996	1	0.003
1997	-	-
1998	1	0.003
1999	-	-
2000	-	-
2001	1	0.003
2002	-	-
2003	-	-
2004	-	-
2005***	59	0.115
2006	-	-
2007	-	-
2008****	24	0.063
2009	5	0.010
2010	-	-
2011	-	-
2012 (I-IV)	-	-

* In fact, at least 1,300 cases confirmed serologically in the great epidemic of 1982–1983 in Zamość Province.
** Cases reported from investigated outbreaks in cattle and secondarily in humans
*** Cases in workers from 2 outbreaks in imported cattle in the border Provinces of Silesia (6 cases) and Małopolska (53 cases).
**** Outbreak in Lublin and Subcarpathian Voivodeship; data registered, but in fact more than 40 human cases.

810 – Leszno, 507 – Leszno, and Dowg – Koszalin. Results of the analysis were compared to 2 *C. burnetii* reference strains – Nine Mile (Montana, USA) and Henzerling (Italy). Among the tested isolates ST18 type was dominated which, together with the ST16 type, is responsible for the acute form of disease. An interesting case was the isolation of *C. burnetii* strain Hum from a 10-year-old Polish boy with mycoplasmosis who had returned from abroad. It was found that this strain showed a high genetic similarity to the *C. burnetii* strain Henzerling. The study demonstrated that all isolates showed high genetic

Table 4. Q fever in animals in Poland 1996–2012, according to OIE (World Organisation for Animal Health – www.web.oie.int).

Years	Region	Cases of infection in animals
1996	No data	1 case* (cattle)
1997	No data	8 outbreaks with 30 cases (cattle)
1998	No data	1 case* (cattle)
1999	No data	1 case* (cattle)
2000	No data	1 case* (cattle)
2001	No data	No data
2002	No data	4 outbreaks with 4 cases (cattle)
2003	No data	2 outbreaks with 2 cases (cattle)
2004	No data	4 outbreaks with 4 cases (cattle)
2005	Lesser Poland Voivodeship	1 outbreak with 2 cases (cattle)
2005	Masovian Voivodeship	2 outbreaks with 66 cases (cattle)
2005	Subcarpathian Voivodeship	1 outbreak with 1 case(cattle)
2005	Silesian Voivodeship	1 outbreak with 1 case (cattle)
2005	Pomeranian Voivodeship	1 outbreak with 1 case (cattle)
2006	Warmian-Masurian Voivodeship	2 outbreaks with 3 cases (cattle)
2007	Subcarpathian Voivodeship	1 outbreak with 1 case (cattle)
2007	Silesian Voivodeship	1 outbreak with 2 cases (cattle)
2008	Lublin Voivodeship (Biłgoraj District)	1 outbreak with 52 cases (cattle)
2008	Subcarpathian Voivodeship (Leżajsk District)	1 outbreak with 199 cases (cattle)
2009	Lublin Voivodeship	1 outbreak with 1 case (cattle)
2009	Opole Voivodeship	1 outbreak with 1 case (cattle)
April 2010	Subcarpathian Voivodeship	1 outbreak with 1 case (cattle)*
June 2010	Greater Poland Voivodeship	1 outbreak with 3 cases (sheep)*
July 2010	Opole Voivodeship	1 outbreak with 11 cases (cattle)**
July 2010	Greater Poland Voivodeship	1 outbreak with 5 cases (sheep)*
October 2010	Masovian Voivodeship	5 outbreaks with 5 cases (cattle)*
October 2010	Podlaskie Voivodeship	6 outbreaks with 7 cases (cattle)*
November 2010	Łódź Voivodeship	2 outbreaks with 3 cases (cattle)*
November 2010	Lubuskie Voivodeship	2 outbreaks with 2 cases (cattle)*
November 2010	Masovian Voivodeship	2 outbreaks with 2 cases (cattle)*
November 2010	Opole Voivodeship	1 outbreak with 1 case (cattle)*
November 2010	Podlaskie Voivodeship	2 outbreaks with 2 cases (cattle)*
November 2010	Pomeranian Voivodeship	2 outbreaks with 4 cases (cattle)*
November 2010	Warmian-Masurian Voivodeship	1 outbreak with 1 case (cattle)*
November 2010	West Pomeranian Voivodeship	3 outbreaks with 4 cases (cattle)*
December 2010	Kuyavian-Pomeranian Voivodeship	2 outbreaks with 3 cases (cattle)*
December 2010	Pomeranian Voivodeship	1 outbreak with 2 cases (cattle)*
December 2010	Silesian Voivodeship	2 outbreaks with 2 cases (cattle)*
December 2010	West Pomeranian Voivodeship	1 outbreak with 1 case (cattle)*
January 2011	Lublin Voivodeship	1 outbreak with 3 cases (cattle)*
January 2011	West Pomeranian Voivodeship	1 outbreak with 1 case (cattle)*
April 2011		1 outbreak with 5 cases (cattle)*
May 2011		1 outbreak with 4 cases (cattle)*
June 2011	Greater Poland Voivodeship	1 outbreak with 1 case (goats)*
July 2011		1 outbreak with 1 case (goats)*
August 2011		1 outbreak with 60 cases (cattle) and 81 cases (goats)*
September 2011		1 outbreak with 1 case (cattle)*
October 2011		No full data 213 destroyed (goats), 24 slaughtered (bovines)
December 2011	No data	1 outbreak with 3 cases (cattle) and 9 cases (goats)*

* Confirmed case, no clinical symptoms. Results of random tests covered by the Regulation of the Minister of Agriculture and Rural Development of 24 June 2010.

diversity. In addition, the 755 strain exhibited a unique MLVA and MST profile, which suggests that it was imported from a territory outside Poland. Applying appropriate sanitary and veterinary eradication methods successfully eliminated this strain from the environment.

In recent years, infection 'imported' by people working on animal farms abroad, especially in the Mediterranean area, have become increasingly significant. The humans suspected of being infected should be submitted to active epidemiological surveillance [57].

The infection may occur as a result of direct animal-animal, animal-human or human-animal contact [9, 14, 15, 49]. Horizontal human-human infections occur very rarely. Several cases of disease transmission through the genital tract and contact with ill pregnant women have been found [6, 8, 9, 15]. The cases of infection among blood recipients, or resulting from bone marrow transplantation [6, 15], as well as cases caused by aerolisation during autopsy, have also been described [6, 8]. For humans, the main sources of *C. burnetii* infection are products derived from animals, such as contaminated wool, meat and milk, animal faeces and urine, as well as dust in the rooms where diseased animals are housed, trucks for their transportation, and contaminated water. The infection occurs mostly via the aerosol route, by inhalation of dust particles contaminated by bacteria. Amniotic fluids, placenta, and foetal membranes are rich sources of the agent (over 1 billion organisms per gram of placenta) [6, 8, 14, 15].

Treatment and prophylaxis. A case definition of Q fever, in accordance with Commission Decision of the European Parliament and European Council of 28 April 2008 (2008/426/WE), specifies the criteria for its diagnosis by clinicians, epidemiologists and laboratory diagnostics.

In the treatment of acute Q fever, the recommended antibiotics are: tetracycline (doxycycline, glicylcycline), macrolides (erythromycin, clarithromycin, and roxithromycin), and quinolones (ciprofloxacin, ofloxacin and trovafloxacin) [49, 66]. Erythromycin is not recommended in severe cases. The treatment should be continued for 7–14 days or longer, depending on the patient's condition [49]. The chronic form requires a much longer period of treatment, even up to 3 years [51]. Sometimes, in case of the long-term antibiotic therapy, cardiac surgery with replacement of damaged heart valves is required.

Recent studies have demonstrated that new generation antibiotics, such as erythromycin-derived telithromycin [67] and tigecycline (glicylcycline), show effective activity against *C. burnetii* [66]. According to Spirydaki *et al.* [66], the strongest activity against *C. burnetii* has been shown by tigecycline and doxycycline, and successively weaker activity by: ofloxacin, trovafloxacin, clarithromycin, linezolid, and ciprofloxacin. Sometimes, the antibiotics can be combined with chloroquine (lysosomal alkalinization mechanism). In some cases (e.g. in children), interferon-γ (IFN-γ) may prove useful for effective treatment [68, 69].

The first vaccine against Q fever containing formalin-killed and ether-extracted *C. burnetii* was available just a few years after the discovery of etiological agent. However, it was more-or-less effective only for patients who were exposed to the microbe by aerosol route [7, 11]. There is only one commercially available human Q-fever vaccine – 'Q-VAX' vaccine (CSL Limited, Parkville, Victoria, Australia). It is widely used in Australia and produced on the basis of formalin-inactivated whole cells of *C. burnetii* strain Henzerling Phase I, which gives high and long-term resistance [70]. In 2001, the Australian National Vaccination Programme' was started, which is unique in the world. Between 2001–2004, more than 40,000 people from high risk groups were vaccinated [71]. Only 94 Adverse Events Following Immunisation (AEFI) have been reported, including one requiring hospitalization. Another vaccine – IND 610 – based on formalin-inactivated whole bacterial cells, has been studied in the US. It has proved to be safe and effective, although it is not achievable at present [6, 15]. The researches on the effectiveness of different vaccines have been performed particularly in the US, the former Soviet Union and Czechoslovakia [9, 11].

Chen *et al.* [72] have selected 7 recombinant proteins on the basis of epitope mapping of antigens CD4 + T cells. The chosen proteins were used as antigens to immunize mice previously vaccinated with PI-WCV vaccine (whole *C. burnetii* phase I) to induce an immune response and antibody production. These studies were designed to find the antigenic similarity of *C. burnetii* and CD4 + T epitopes, which might be used to develop vaccines against *C. burnetii*. Other studies on recombinant vaccines containing protein subunits were unsuccessful [73, 74]. Beare *et al.* [75] used nitrocellulose microarrays to test recombinant *C. burnetii* antigens with regard to their reaction with antibodies produced in response to infection or immunization. The authors obtained positive result for approximately 50 proteins that might be used in the future to produce vaccines against Q fever.

In the Netherlands, Hogerwerf *et al.* [76] experimentally applied a vaccine called Coxevac (phase I containing inactivated Nine Mile *C. burnetii* strain) in sheep and dairy goats, obtaining limitation of infections and abortions, as well as decreased spread of pathogen in the environment. A vaccine known as Chlamyvax FQ, composed of inactivated *C. burnetii* phase II, has also been tested on animals, but without positive results [77]. A chloroform-methanol residue (CMR) vaccine, tested in the US, provided immunity in animals, including primates, in cases of infection by the aerosol route; however, it caused severe side-effects [8, 9, 11].

The four-year studies by Astobiza *et al.* (2007–2011) on the effectiveness of vaccine consisting of phase I *C. burnetii* in the sheep herd with confirmed infection, showed that use of the vaccine inhibited further spread of the pathogen in the herd for the last 2 years, despite its persistence in the environment [78].

In the non-specific prevention of the disease in animals, the most important measure is to quarantine animals introduced into the herd, and the eradication of sick animals in the case of disease occurrence, and the isolation of microbes. Q fever is not a disease treated *ex officio*. Milk from sick animals is not suitable for human consumption; however, after submition to pasteurization it may be used in animal feeding on the farm [23]. Detailed data concerning proceedings in the event of Q fever emergence in animals include guidelines of the Chief Veterinary Officer [48].

The prevention of Q fever in humans relies on maintaining special caution and personal hygiene rules in contact with animals. In the case of emergence of the disease among animals, special medical care should be provided for the persons on the farm in whom the presence of antibodies against *C. burnetii* should be monitored. In cases of confirmed contact with infected animals, it is advisable to use antibiotics

prophylacticly [35, 57]. In epidemic areas, the blood sampling from donors must be stopped. Q fever is a typical biological agent of occupational hazard [79].

LABORATORY DIAGNOSTICS

Culture methods. In most cases, the isolation of living strains of *C. burnetii* is performed with the use of *in vitro* cultures of cell lines, such as Vero cell line (African Green Monkey Kidney epithelial), also known as MGB cell line, BHK – 21 (hamster kidney fibroblast), L – 929 (murine fibroblast), HEL (Human Cervical epithelial, and CHO (Chinese hamster ovary fibroblast), J774A.1 (murine macrophage-like) [37].

Recently, an artificial medium, acidic citrate cysteine medium (ACCM), for culturing *C. burnetii* has been introduced [80]. Acidic pH (4.75) and low concentration of oxygen (2.5%) imitate conditions that are characteristic for PV vacuoles in cells. Log phase was observed after 6 days of culturing.

Serological methods. Q fever as an infectious disease is difficult to diagnose. Preliminary diagnostics are mainly based on clinical symptoms, direct contact with animals, and on serological results [7, 63].

Two forms of *C. burnetii* can be detected by serological methods, such as IFA (Indirect immunofluorescence assay) and ELISA (Immunoenzymatic assay). The first form detected is the virulent phase, known as phase I, which possess smooth LPS. The second form is phase II, which possess rough LPS [7, 81]. Defining the titers of phase I and phase II antibodies allows distinguishing the acute form from the chronic form of Q fever, and is a predictor indicating the evolution of the acute form to the chronic form [7, 23, 81]. The need for clinical observation, using for example, 2-D echocardiography, and serological monitoring of convalescents who showed the acute form of the disease, for many years has been indicated as essential in terms of the possibility to develop the chronic form in these patients [23, 35, 82]. Currently, this postulate has been recognized by ECDC as a standard procedure [83].

To define the titer of antibodies against *C. burnetii*, IFA and ELISA are usually used. In IFA, antigens of phase I and phase II are applied, which are produced in fibroblasts of infected mice and murine epithelial placenta cells [36]. This method allows obtaining high quantities of highly specific antigens which can be used for the detection of IgG, IgM, and IgA antibodies [36]. Peter *et al.* [84] and Cowley *et al.* [85] showed that ELISA and IFA can be used in routine serodiagnosis of Q fever, although ELISA is more labour- and time-consuming, and requires highly qualified personnel [36].

In acute infections, antibody response to *C. burnetii* phase II antigen is predominant and is higher than phase I antibody response. In chronic infections, an increase of the phase I IgG titer is observed, which is often much higher than phase II IgG.

The gold standard of serological tests for diagnosis of acute Q fever is the indirect immunofluorescence assay with the use of *C. burnetii* antigen, which is performed on paired serum samples to demonstrate a significant (4-fold) rise in antibody titers. In most cases of Q fever, the first IgG IFA titer is typically low, or 'negative', and the second typically shows a significant (4-fold) increase in IgG antibody levels. Antibodies concentration for phase II above 1:64 for IgM and above 1:256 for IgG are usually defined as positive [44].

For many years, the serological diagnosis of humans was based on 2 highly specific tests: complement fixation test (CSF) and microagglutination test in phases I and II, performed simultaneously [19, 23, 60]. Other methods, such as the complement fixation test, western-blotting, microagglutination tests and radioimmunological assays, have also been applied in the diagnostics of Q fever [36].

Molecular diagnostics – PCR method. Amplification of defined fragments of DNA with the PCR method has been used for the detection of *C. burnetii* genetic material. Designing primers and probes, highly specific for *C. burnetii* genes, is nowadays an easy task in science, enabling adaption of the PCR method for diagnostic purposes, and giving the possibility to detect genetic material of the patogen in various samples. PCR is regarded as a highly sensitive method in comparison to standard, serological methods, which is why it has wide application in screening tests [36], and has been used for identification of *C. burnetii* in samples of various origin, including clinical samples (blood, biopsy, liver and placenta), environmental samples, animal faeces and tissues of ticks [86].

Currently, many different primer sequences for detection of *C. burnetii* is available in the literature [86]. For preliminary detection of *C. burnetii* in environmental samples, insertion elements, such as *IS1111* and *htpAB* are mainly used, because they can occur in bacterial genome in many copies, 19 and 20, respectively. These properties of target sequences enhance the sensitivity of the PCR reaction, allowing detection of even a single cell in a sample [87]. *C. burnetii* possess 29 insertion elements: 21 copies of *IS1111*, 5 copies of *IS30* and 3 copies of *ISA1*. Insertion sequences are present in bacterial chromosomes, but not in plasmids [88].

For identification of *C. burnetii*, other improved PCR-based methods have also been developed. Nested PCR and real time PCR with TaqMan probes have been applied to detect chromosomal genes (*com1 and htpB*), plasmid genes and transposase-encoding genes located in the insertion elements of bacteria. However, in quantitative analyses, the insertion elements are rarely used due to their variable and not fully defined number of copies [87].

In phylogenetic analyses, which are important for epidemiological investigations, 2 methods based on PCR and sequencing are used: Multilocus Variable Number Tandem Repeats Analysis and Multi Spacer Typing. MLVA is an improved Variable Number Tandem Repeats (VNTR) method, in which series of tandem repeats are flanked by designed primers and multiplied by PCR [27]. Tandem repeats can be located in different parts of the genome, and also occur in many copies [86]. MLVA has been successfully used for the molecular typing of *C. burnetii* [27, 65, 89, 99], which is especially important in epidemiological investigations.

Another molecular method allowing determination of similarities between *C. burnetii* strains is MST, which is based on the sequencing of intergenic spacers [28, 90, 91, 92], since in the spacer regions a single nucleotide polymorphism or insertion/deletion mutations can occur randomly. The profiles obtained by this method can be easily compared using web sites, where 34 different MST profiles have been

already described (see: http://ifr48.timone.univ-mrs.fr/MST_Coxiella/mst).

Microarray constitutes another modern genetic tool used in the analyses of bacterial genes and genomes [29]. The use of microarrays enabled the description of 5 different polymorphisms in *C. burnetii* genomes, and some phylogenetic analyses were also performed using this technique. Thanks to the comparitive genome hybridization method (CGH), it has been shown that the strains belonging to I, II and III genomic group and carrying QpH1 plasmid are genetically different microorganisms. The results of the presented study show that strains belonging to genomic group I are the ancestors of the group III, while strains from genomic group III are the ancestors of the genomic group II [29].

***C. burnetii* as a biological weapon.** *C. burnetii* can be used in bioterrorist attacks via aerosol, contaminated environment, food and water [6, 15]. It has been suspected that it was used as biological warfare against German soldiers during World War II in Serbia and southern Yugoslavia, where suspicious outbreaks were noted [11].

In the Amercian Offensive Program of 1942, *C. burnetii* was taken into account as a (possible) biological agent which could be used for the production of biological weapons.

In 1955, volunteers from the Seventh-Day Adventists Church were exposed to *C. burnetii* aerosol in the so-called 'Whitecoat' program and were subjected to therapeutical tests. Before the outbreak in World War II, the Russians also propably produced *C. burnetii* for offensive purposes. In 1995, a religious group, The Highest Truth (Aum Shinrikyo), carried out the terrorist attack on Tokyo underground using Sarin. This incident proved the effectiveness of the use of biological agents, including *C. burnetii*, for the purpose of biological attacks by terrorists [6]. According to the World Health Organization (WHO), the aerolization of 50 kg of *C. burnetii* in an urbanized area with a population of about 500,000 inhabitants, could cause 125,000 acute, 900 chronic, and 150 fatal cases [9].

According to the CDC *C. burnetii* belongs to group B pathogens in terms of biothreat. Even though an attack with *C. burnetii* would not cause fatal consequences, unlike *Yersinia pestis* or *Bacillus anthracis*, it would eliminate infected people from active life for the duration of their treatment. *C. burnetii* shows many useful features with regard to military applications:

– it is extremely resistant to environmental factors;
– it is remains virulent during long periods of storage;
– it is easy to spray and produces quick and long-term overpowering effects.

C. burnetii is also a pathogen, which is unusually difficult to eliminate from the environment, a process that is very time-consuming. In the case of natural occurring outbreaks (natural focus, nidality focus) elimination of the microorganism is simply impossible. The need for long-term therapy, drastically decreased military ability of infected soldiers and reduced ability of the civilian population to work, as well as the possibility of relapse or transformation into the chronic form of the disease, causing serious health consequences, are unfotunatelly the biggest 'advantages' of *C. burnetii* as a potential bioterrorism agent.

CONCLUSION

In this article the aspects regarding molecular characteristic of *C. burnetii* and mechanism of pathogenesis were described. New issues in the treatment, prophylaxis and diagnosis of disease caused by *C. burnetii* were also presented. Additionally, animal and human Q fever prevalence in Poland was reviewed. Possibility of using *C. burnetii* as a biological agent in bioterrorist attack was discussed.

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