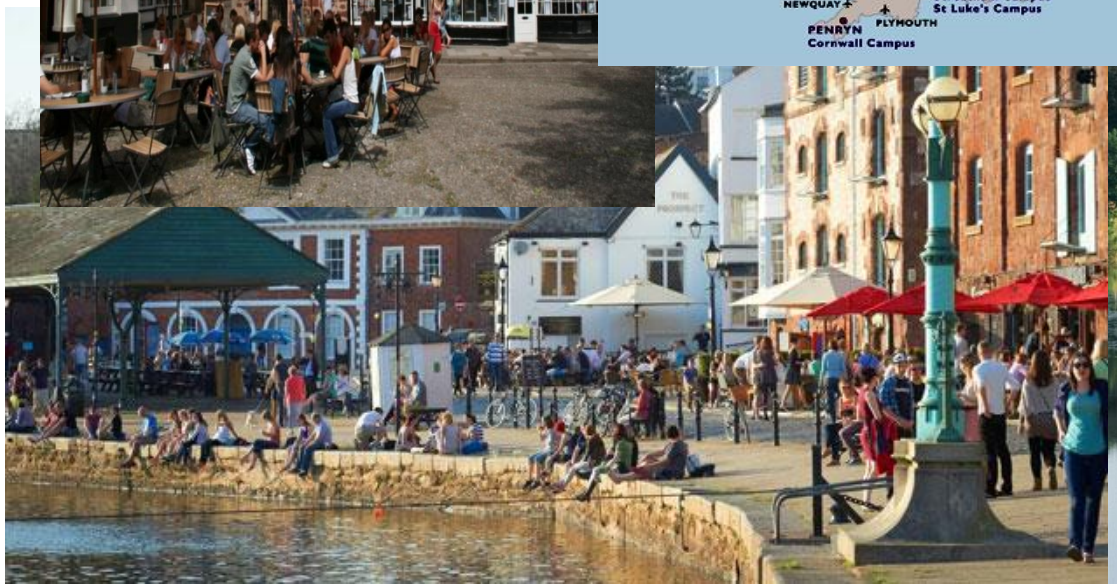
Q is for query
V is for vaccine

- Dr Steve Michell
- Dr Steve Porter
- Dr Alan Brown
- Prof Rick Titball



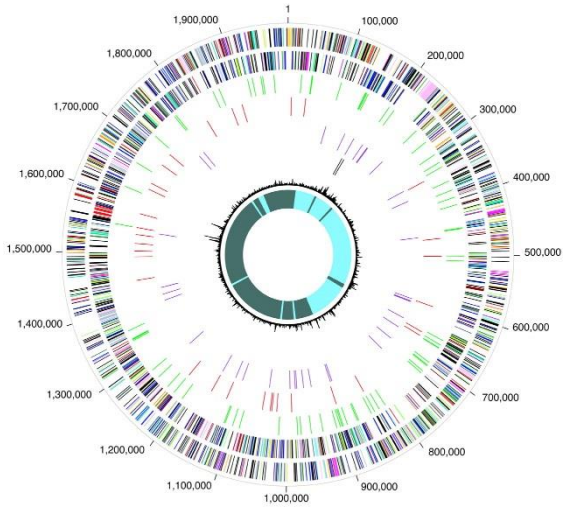
Q-fever

- First identified in Australia in 1935
- “Q” is for query
- But at that time the etiological agent could not be isolated

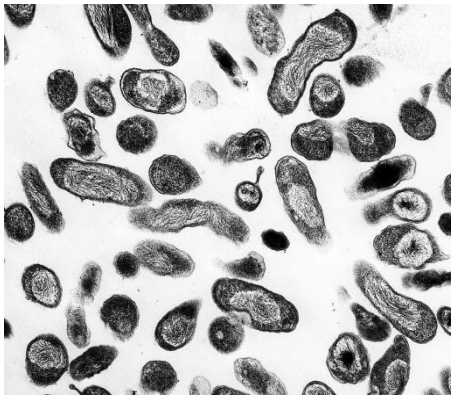
- Caused by the bacterium *Coxiella burnetii* (1938)

Coxiella burnetii

- Gram negative, intracellular bacteria
- Obligate intracellular pathogen
- Zoonotic pathogen carried predominantly by goats, sheep and cattle
- Genome size – 1.9 – 2.2 mbp
  - plasmids QpH1, QpRS, QpDG, QpDV
- Six genomic groups proposed, based on restriction endonuclease digests of genomic DNA



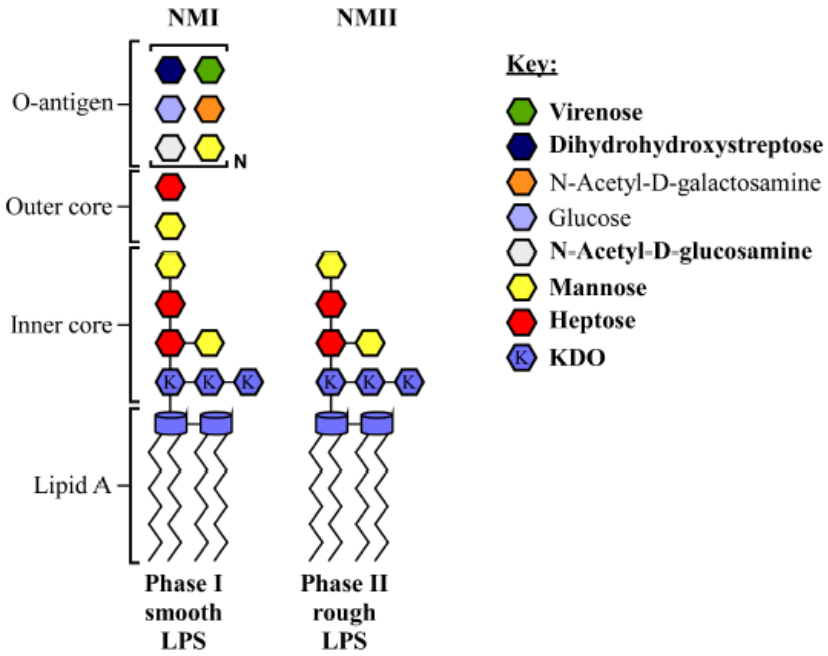
Genome of *C. burnetii* RSA492 -(Seshadri *et.al.,* 2003)



Electron micrograph depicting small and large colony variants: Rocky mountain Labs

Phase variation

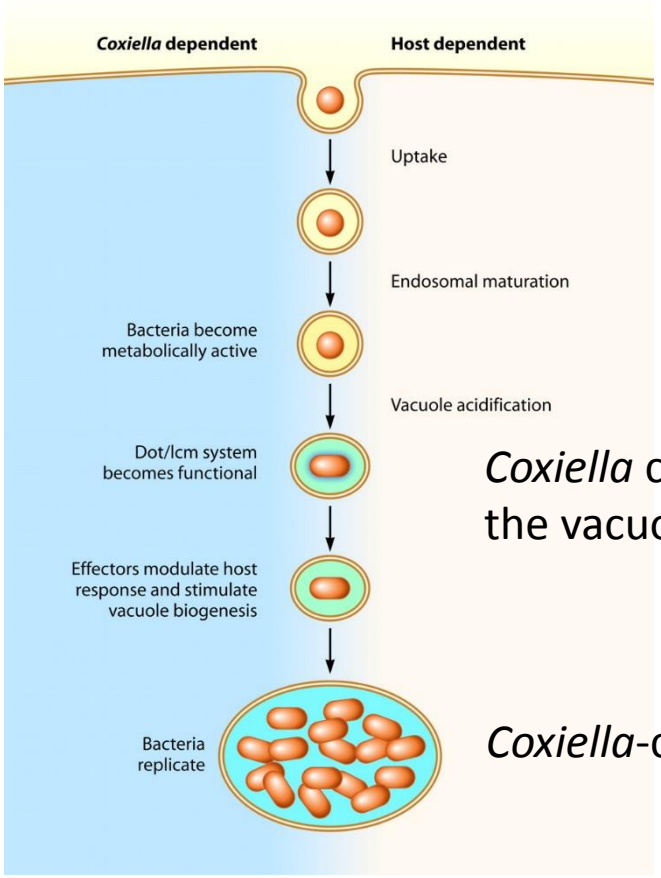
- Phase I – virulent, CL3 e.g. Nine Mile I (NMI)
  - Complete LPS with O antigen sugars L-virenose and dihydrohydroxystreptose
- Phase II – attenuated, CL2 e.g. Nine Mile II (NMII)
  - Truncated LPS without O antigen
  - Isolated following multiple passage in egg yolk sacs, tissue culture or axenic media
- Genetic mechanism recently elucidated
  - Accumulation of 14 mutations in 11 predicted LPS-associated genes



C. burnetii LPS forms: Beare *et al*. 2018

Host and pathogen functions important for *C. burnetii* infection.

T4SS activated



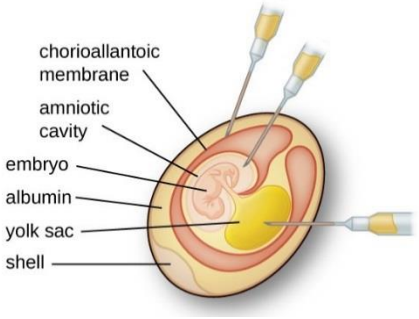
Coxiella can only replicate when the vacuole becomes acidified

Coxiella-containing vacuole (CCV)

Hayley J. Newton, and Craig R. Roy mBio 2011; doi:10.1128/mBio.00226-11

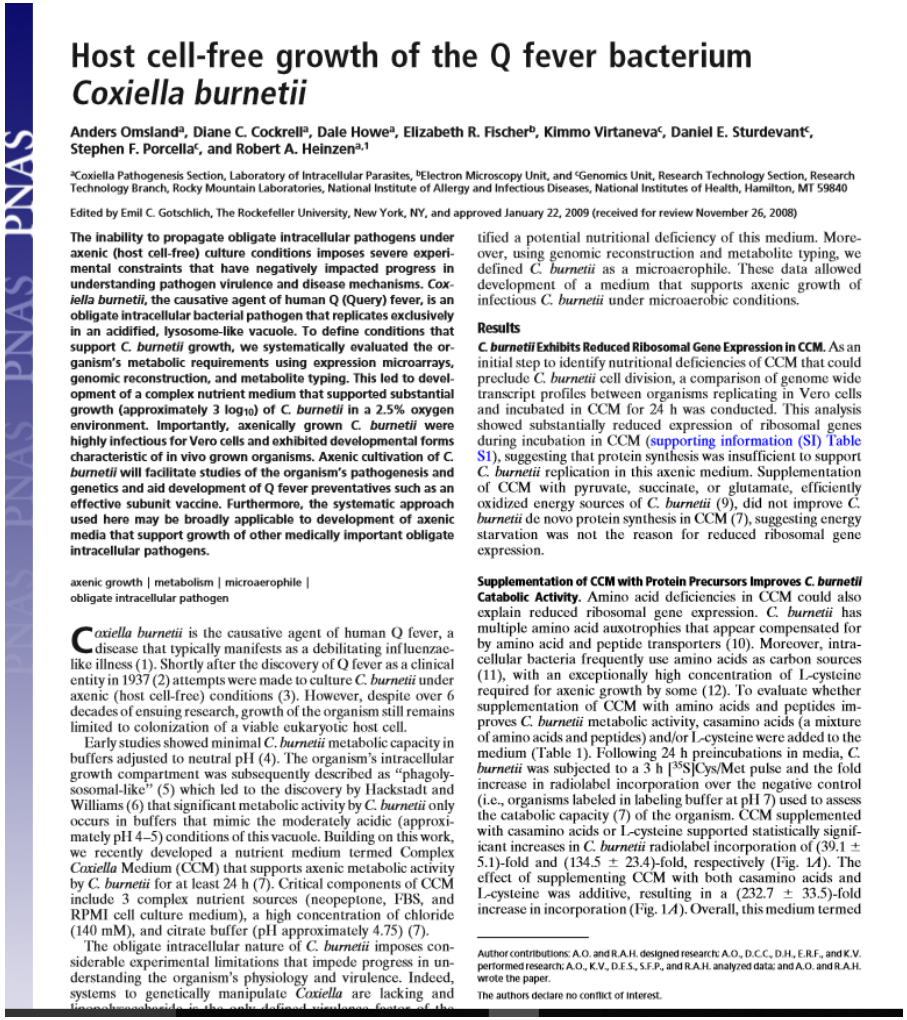
Growth of *C. burnetii*

- Growns well in yolk sac of chick embryos



- Can be grown in cell cultures e.g. Vero, macrophage cell lines.

Cell-free growth of *C. burnetii*



Host cell-free growth of the Q fever bacterium *Coxiella burnetii*

Anders Omsland[a], Diane C. Cockrell[a], Dale Howe[a], Elizabeth R. Fischer[b], Kimmo Virtaneva[c], Daniel E. Sturdevant[c], Stephen F. Porcella[c], and Robert A. Heinzen[a,1]

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Edited by Emil C. Gotschlich, The Rockefeller University, New York, NY, and approved January 22, 2009 (received for review November 26, 2008)

The inability to propagate obligate intracellular pathogens under axenic (host cell-free) culture conditions imposes severe experimental constraints that have negatively impacted progress in understanding pathogen virulence and disease mechanisms. *Coxiella burnetii*, the causative agent of human Q (Query) fever, is an obligate intracellular bacterial pathogen that replicates exclusively in an acidified, lysosome-like vacuole. To define conditions that support *C. burnetii* growth, we systematically evaluated the organism's metabolic requirements using expression microarrays, genomic reconstruction, and metabolite typing. This led to development of a complex nutrient medium that supported substantial growth (approximately 3 $\log_{10}$) of *C. burnetii* in a 2.5% oxygen environment. Importantly, axenically grown *C. burnetii* were highly infectious for Vero cells and exhibited developmental forms characteristic of in vivo grown organisms. Axenic cultivation of *C. burnetii* will facilitate studies of the organism's pathogenesis and genetics and aid development of Q fever preventatives such as an effective subunit vaccine. Furthermore, the systematic approach used here may be broadly applicable to development of axenic media that support growth of other medically important obligate intracellular pathogens.

axenic growth | metabolism | microaerophile | obligate intracellular pathogen

Coxiella burnetii is the causative agent of human Q fever, a disease that typically manifests as a debilitating influenzae-like illness (1). Shortly after the discovery of Q fever as a clinical entity in 1937 (2) attempts were made to culture *C. burnetii* under axenic (host cell-free) conditions (3). However, despite over 6 decades of ensuing research, growth of the organism still remains limited to colonization of a viable eukaryotic host cell.

Early studies showed minimal *C. burnetii* metabolic capacity in buffers adjusted to neutral pH (4). The organism's intracellular growth compartment was subsequently described as "phagolysosomal-like" (5) which led to the discovery by Hackstadt and Williams (6) that significant metabolic activity by *C. burnetii* only occurs in buffers that mimic the moderately acidic (approximately pH 4–5) conditions of this vacuole. Building on this work, we recently developed a nutrient medium termed Complex *Coxiella* Medium (CCM) that supports axenic metabolic activity by *C. burnetii* for at least 24 h (7). Critical components of CCM include 3 complex nutrient sources (neopeptone, FBS, and RPMI cell culture medium), a high concentration of chloride (140 mM), and citrate buffer (pH approximately 4.75) (7).

The obligate intracellular nature of *C. burnetii* imposes considerable experimental limitations that impede progress in understanding the organism's physiology and virulence. Indeed, systems to genetically manipulate *Coxiella* are lacking and

tified a potential nutritional deficiency of this medium. Moreover, using genomic reconstruction and metabolite typing, we defined *C. burnetii* as a microaerophile. These data allowed development of a medium that supports axenic growth of infectious *C. burnetii* under microaerobic conditions.

Results

C. burnetii Exhibits Reduced Ribosomal Gene Expression in CCM. As an initial step to identify nutritional deficiencies of CCM that could preclude *C. burnetii* cell division, a comparison of genome wide transcript profiles between organisms replicating in Vero cells and incubated in CCM for 24 h was conducted. This analysis showed substantially reduced expression of ribosomal genes during incubation in CCM (supporting information (SI) Table S1), suggesting that protein synthesis was insufficient to support *C. burnetii* replication in this axenic medium. Supplementation of CCM with pyruvate, succinate, or glutamate, efficiently oxidized energy sources of *C. burnetii* (9), did not improve *C. burnetii* de novo protein synthesis in CCM (7), suggesting energy starvation was not the reason for reduced ribosomal gene expression.

Supplementation of CCM with Protein Precursors Improves *C. burnetii* Catabolic Activity. Amino acid deficiencies in CCM could also explain reduced ribosomal gene expression. *C. burnetii* has multiple amino acid auxotrophies that appear compensated for by amino acid and peptide transporters (10). Moreover, intracellular bacteria frequently use amino acids as carbon sources (11), with an exceptionally high concentration of L-cysteine required for axenic growth by some (12). To evaluate whether supplementation of CCM with amino acids and peptides improves *C. burnetii* metabolic activity, casamino acids (a mixture of amino acids and peptides) and/or L-cysteine were added to the medium (Table 1). Following 24 h preincubations in media, *C. burnetii* was subjected to a 3 h [^{35}S]Cys/Met pulse and the fold increase in radiolabel incorporation over the negative control (i.e., organisms labeled in labeling buffer at pH 7) used to assess the catabolic capacity (7) of the organism. CCM supplemented with casamino acids or L-cysteine supported statistically significant increases in *C. burnetii* radiolabel incorporation of (39.1 ± 5.1)-fold and (134.5 ± 23.4)-fold, respectively (Fig. 1*A*). The effect of supplementing CCM with both casamino acids and L-cysteine was additive, resulting in a (232.7 ± 33.5)-fold increase in incorporation (Fig. 1*A*). Overall, this medium termed

Author contributions: A.O. and R.A.H. designed research; A.O., D.C.C., D.H., E.R.F., and K.V. performed research; A.O., K.V., D.E.S., S.F.P., and R.A.H. analyzed data; and A.O. and R.A.H. wrote the paper.

The authors declare no conflict of interest.

- Map transcriptome
- Predict nutritional requirements from metabolic pathway profiling
  - Auxotrophic for many amino acids
- Consider the niche in which the bacterium replicates - the Coxiella containing vacuole (CCV) an acidified endosome
  - Low pH (~4.5)
  - Low oxygen levels (2.5%)

ACCM-2 growth medium

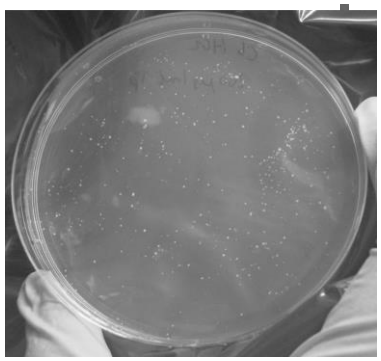
Salts, Vitamins, Minerals & Trace Elements
- calcium chloride dihydrate (26 mg/L)
- citric acid (5140 mg/L)
- citric acid trisodium anhydrous (8320 mg/L)
- iron (II) sulfate hydrate (3 mg/L)
- magnesium chloride anhydrous (190 mg/L)
- potassium phosphate monobasic anhydrous (1000 mg/L)
- sodium chloride (14560 mg/L)

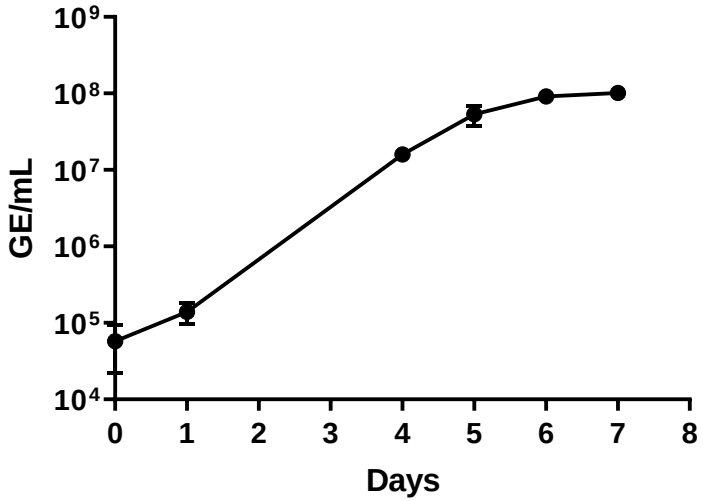
Amino Acids & Supplements
- L-Cysteine hydrochloride monohydrate (527 mg/L)

Rich Supplements
- casamino acids (5000 mg/L)
- tryptone (casein peptone) (200 mg/L)

Other Additives
- methyl-b-cyclodextrin (2000 mg/L)

- pH adjusted to 4.75
- 2.5% O_2





Q-fever in livestock

- A disease of concern in livestock, especially sheep, goats and cattle
- But infection of other wild animals reported
- Vector borne transmission via ticks
- Infection is a cause of morbidity, and abortion in pregnant animals

Global distribution of disease in animals

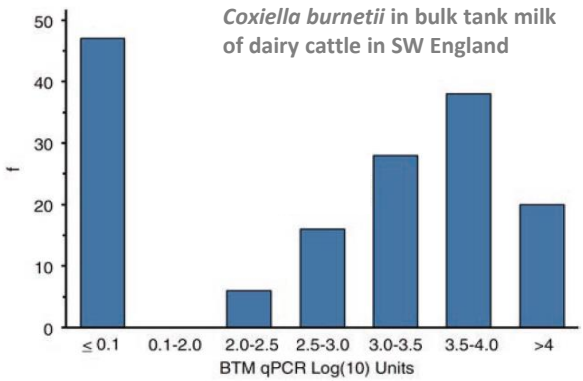
- Found globally in most counties (except New Zealand)

Seroprevalence in animals in the UK:

13-29 % of cattle
9-12 % of sheep
9 -26 % of goats
53% of rats
41% of foxes
61% of cats

C. burnetii in milk

- Sampling bulk milk in the UK reveals that 80% of herds are infected (Velasova M, *et al*. 2017)
  - confirmatory testing found 29% of herds were PCR positive

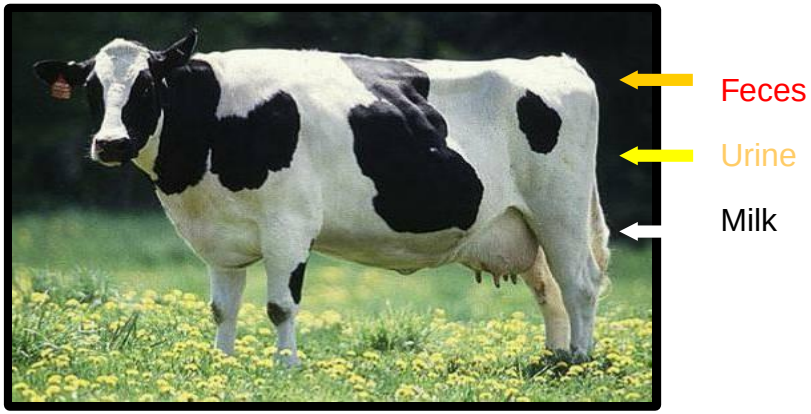


Valergakis GE *et al.* (2012) *Vet Rec*, **171**(6):156

- Pasteurisation kills bacteria, but an emerging trend is to drink untreated milk

Q-fever in humans

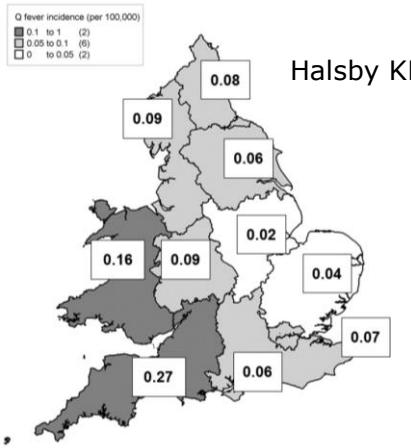
- **Experimental disease.** Evidence of infectious dose from human volunteer studies in the USA in the 1950s (Brooke *et al*., 2013)
  - ID_{50} dose 1 infectious unit
  - Dose for 50% illness = 5 infectious units
- **Naturally occurring disease**
  - By exposure to infected animals or animal products
  - By the inhalation of bacteria
  - By ingestion of contaminated foodstuffs



Q-fever in humans

- **Acute**
  - Acute disease is often characterised by flu-like symptoms
  - High fever, retro-orbital headache, pneumonia
  - 1-2% of cases develop a pneumonia
- **Chronic**
  - 5-15% of cases become chronic
  - Endocarditis, hepatitis and chronic fatigue
  - Miscarriage or low birth weight in pregnant women
- **Asymptomatic**
  - 50% of infections
  - In the UK 27% of farmers and 10% of the general population show signs (antibodies) indicating previous exposure

Human disease in the UK



Halsby KD *et al*. (2017) *Vet Sci*, **4**:28

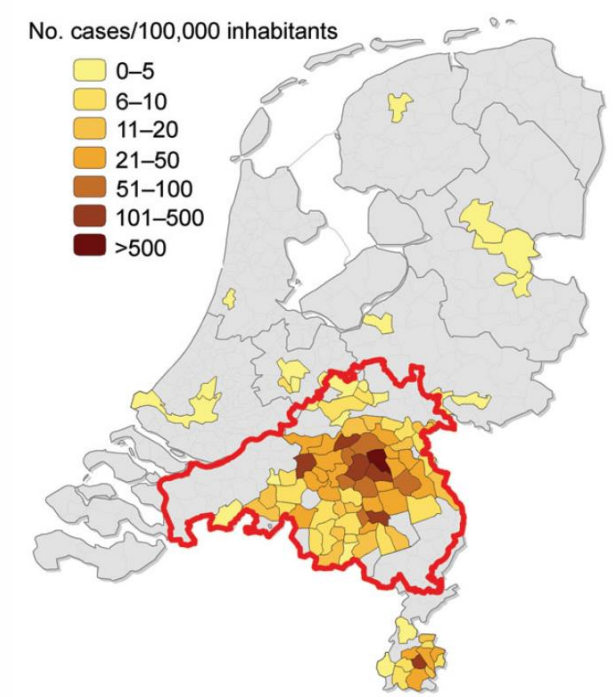
In 2007, an outbreak of Q fever occurred in Cheltenham, with **30 confirmed or probable human cases** identified. An investigation identified windborne spread of *Coxiella burnetii* from nearby sheep farms as the most likely source of infection.

In 2002, an outbreak of Q fever occurred in South Wales at a cardboard manufacturing plant, with **95 cases** identified. The offices were undergoing renovation work and the outbreak may have been caused by aerosolisation of *Coxiella burnetii* spore-like forms during drilling into contaminated straw board.

In 2006, the largest outbreak of Q fever in Scotland occurred at a co-located slaughterhouse and cutting plant, with **110 cases**. Preliminary investigations pointed to the sheep lairage being the potential source of exposure to the infective agent.

The Netherlands outbreak

- Largest outbreak 2007-2010
- more than 4,000 human cases in the Netherlands; required euthanizing 50,000 goats.



Human cases Jan-June 2009. Area in red shows mandatory Sheep and goat vaccination area

Hogerwerf *et al.*, (2011) Emerg Infect Dis 17:379-386

Q-fever and chronic disease

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British soldier sues Army over Q fever chronic fatigue

By Clive Coleman
Legal correspondent, BBC News

21 January 2019 Share

WAYNE BASS

Wayne Bass served in Helmand in 2011

A former soldier is suing the Ministry of Defence after contracting Q fever in Afghanistan.

- Most cases present as Q-fever endocarditis.
- Evidence that 10-15% of acute cases, progress to chronic fatigue, lasting 5-10 years (Bewley KR. Comp Med. 2013 63(6):469–476)

Q-fever in Thailand

PLOS ONE

RESEARCH ARTICLE

Seropositivities against brucellosis, coxiellosis, and toxoplasmosis and associated factors in pregnant women with adverse pregnancy outcomes: A cross-sectional study

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☯ These authors contributed equally to this work.
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Abstract

Background

Brucellosis, coxiellosis, and toxoplasmosis can be transmitted from infected ruminants to pregnant women and may induce adverse pregnancy outcomes; however, there are to date few studies. This study aimed to examine the seropositivities of immunoglobulin G (IgG) against those three pathogens among pregnant women with adverse pregnancy outcomes, and to explore the associated factors.

Methods

A cross-sectional study was conducte
mon. A total of 105 pregnant Thai wor
serum samples collected at first anter
June 2015 to June 2016 were include
Toxoplasma gondii, and *Coxiella burn*

OPEN ACCESS

Citation: Kledmanee K, Liabsuetrakul T, Sretirutchai S (2019) Seropositivities against brucellosis, coxiellosis, and toxoplasmosis and associated factors in pregnant women with adverse pregnancy outcomes: A cross-sectional study. PLoS ONE 14(5): e0216652. https://doi.org/10.1371/journal.pone.0216652

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Acute Q Fever Case Detection among Acute Febrile Illness Patients, Thailand, 2002–2005

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Abstract. Acute Q fever cases were identified from a hospital-based acute febrile illness study conducted in six community hospitals in rural north and northeast Thailand from 2002 to 2005. Of 1,784 participants that underwent *Coxiella burnetii* testing, nine (0.5%) participants were identified in this case-series as acute Q fever cases. Eight case-patients were located in one province. Four case-patients were hospitalized. Median age was 13 years (range: 7–69); five were male. The proportion of children with acute Q fever infection was similar to adults ($P = 0.17$). This previously unrecognized at-risk group, school-age children, indicates that future studies and prevention interventions should target this population. The heterogeneity of disease burden across Thailand and milder clinical presentations found in this case-series should be considered in future studies. As diagnosis based on serology is limited during the acute phase of the disease, other diagnostic options, such as polymerase chain reaction, should be explored to improve acute case detection.

BACKGROUND

Q fever is caused by the intracellular, gram negative bacteria, *Coxiella burnetii*.[1] Transmission primarily occurs through the inhalation of aerosolized spore-like particles originating from animal blood, birthing fluids, and/or excreta.[2] Although commonly asymptomatic or occasionally marked by reproductive issues in the ruminant reservoir, it can be present in humans as an influenza-like illness, pneumonia, and/or hepatitis, with a case fatality rate below 2%.[3,4] Most (> 90%) patients promptly eradicate the bacterium. However, months to years later, persistent focalized infections (previously referred to as chronic Q fever) can be diagnosed in 1–5% of those having presented with primary infection.[5–8] Persistent focalized infections primarily include cardiovascular infections, which can be fatal if not treated with appropriate antibiotics as well as surgery in aortic infections.[1,9–11]

Although variable antibody kinetics have been described, seroconversion for *C. burnetii* occurs around 7–15 days but can be delayed for as long as 6 weeks.[1,3] Diagnosis is most

The objective of our investigation was to determine the frequency of serologically confirmed acute Q fever infection among febrile patients presenting to district hospitals in rural Thailand and identify at-risk groups. The secondary objective was to assess the utility of different serological criteria for diagnosing acute Q fever to better understand potential limitations of existing diagnostic testing options and to inform future Q fever studies and clinical efforts in Thailand.

METHODS

From 2002 to 2005, an AFI study, as a part of a broader U.S. Centers for Disease Control and Prevention Global Disease Detection AFI network, was conducted in three provinces in north and northeast Thailand: Chiang Rai (2002–2005), Khon Kaen (2002–2004), and Nakhon Phanom (2004–2005), in two community hospitals in each province. Study staff enrolled outpatients and inpatients using the following criteria: age greater than 6 years, presenting within 2 weeks of fever onset, confirmed temperature > 38°C, and resident of the study site

- Febrile patients in community hospitals without obvious cause of infection
- 0.5% of these patients showed rising antibody titres consistent with Q-fever infection

Infection models

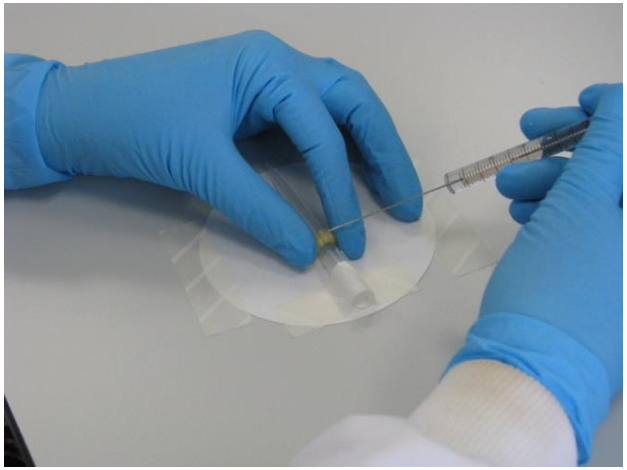
- **Mice**
  - Immunocompetent mice show minimal signs of disease
  - SCID mice develop chronic disease caused by phase 1 strains
- **Guinea pigs**
  - Acute infection caused by phase 1 strains
- **Non-human primates**
  - Acute infection caused by phase 1 strains

lowest	0	none
	1	mild
	2	moderate
highest	3/4	marked/severe

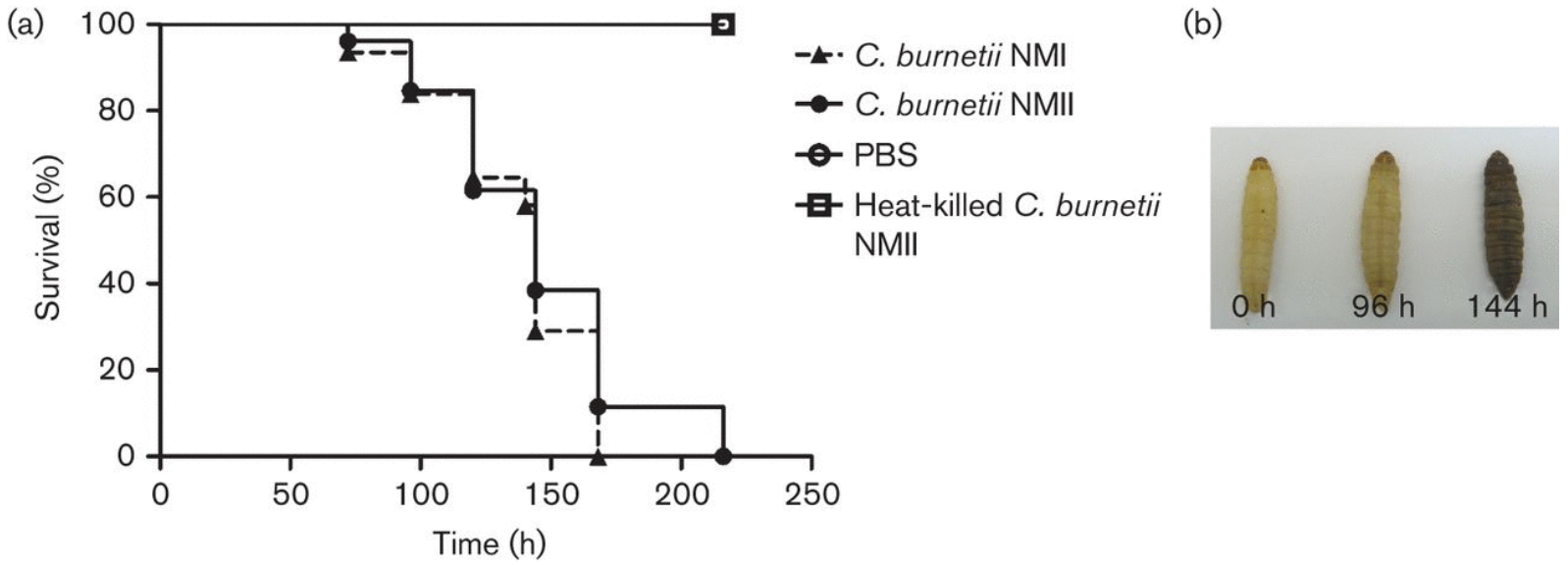
				Genomic Group													
				I			II	III	IV				V		VI		
Model	Route	Dose	Signs of disease	NMI	Ohio	African	Henzerling	CbBEC1	Idaho_Goat	CbBEB1	MSU_Goat	P_Q238	P_Q173	AuQ01	G_Q212	S_Q217	Dugway_7D77	Dugway_7E65	Dugway_5J108
Guinea Pig	i.p.	10^5	Body temperature increase	3	3		2		2		1	1			0	2	0	0	
			Body weight loss	3	3		0		3		1	0			0	0	0	0	
			Splenomegaly at 14 days p.i.	3	2		2		0		1	1			0	2	0	0	

Galleria mellonella as an infection model

- Easy to inject via prolegs
- Incubation at 37°C
- Low rearing costs
- immune system shows similarities with mammalian innate immune system



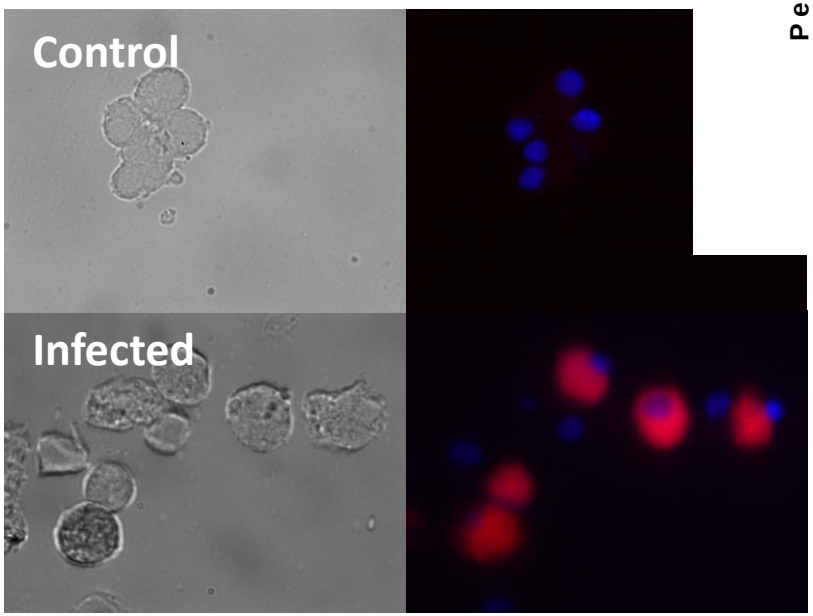
G. mellonella infection with *C. burnetii*

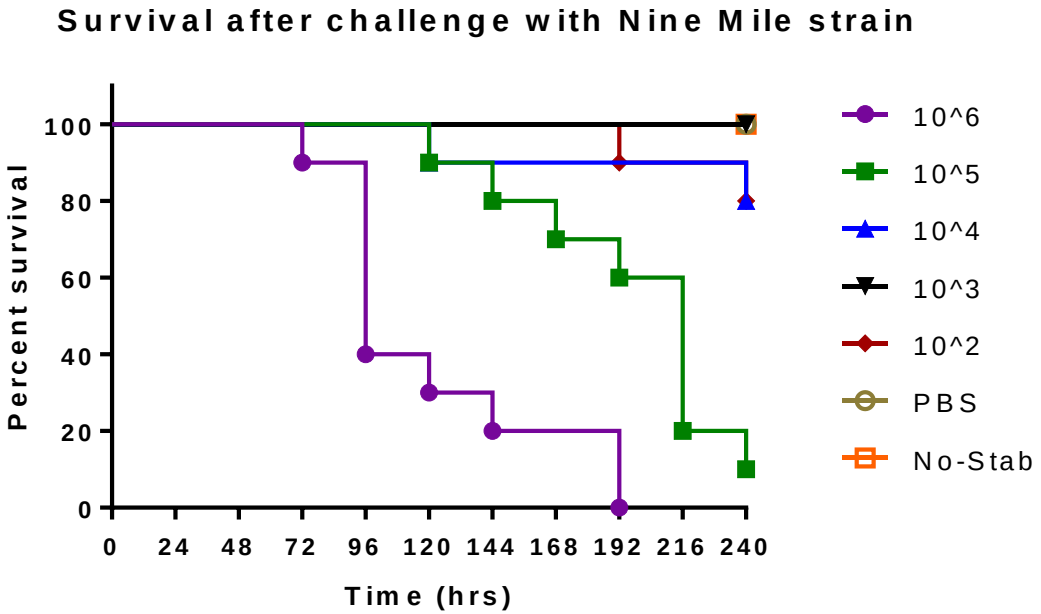


Norville *et al.*, Microbiology. 2014 160:1175-81

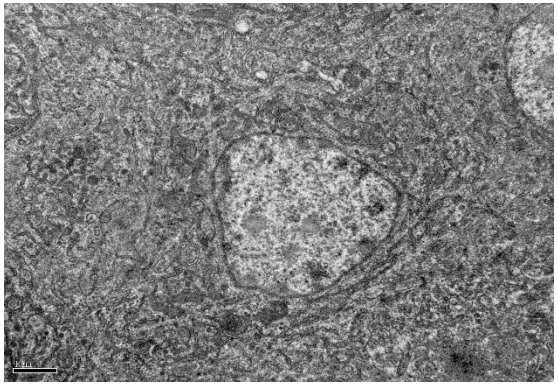
G. mellonella infection with *C. burnetii*

- LD_{50} = 1.19x10^4 GE/mL

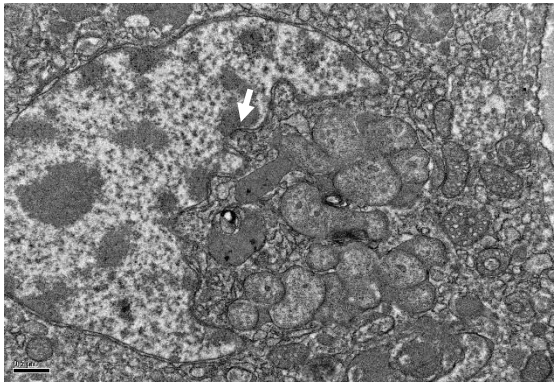




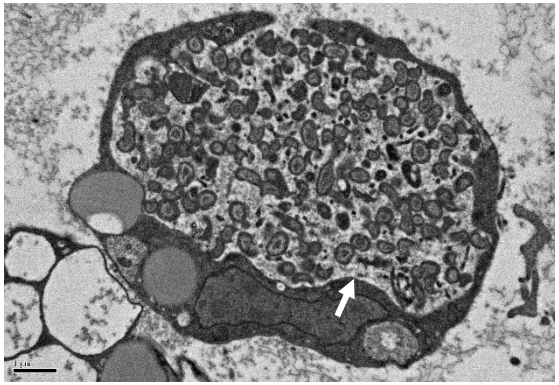
C. burnetii resides within haemocytes



Uninfected control



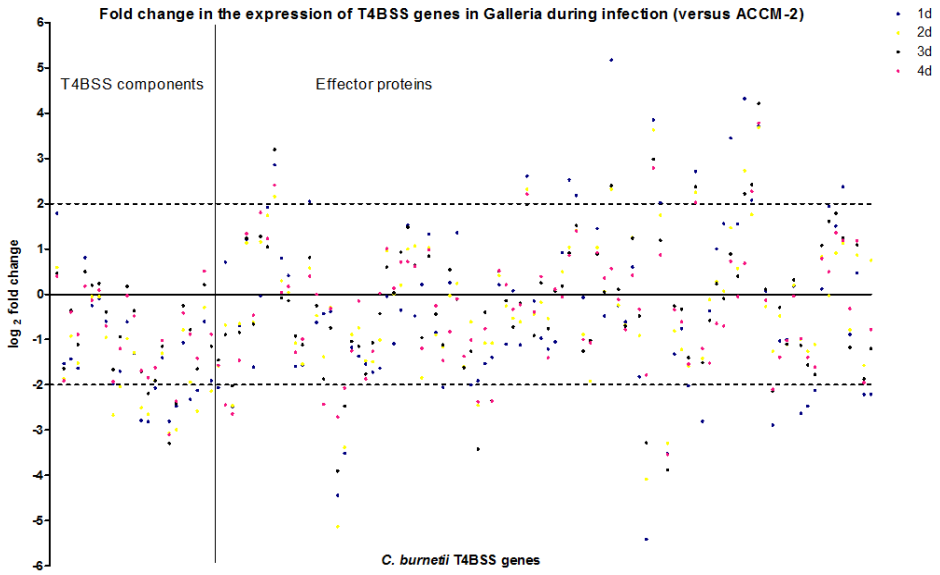
48hrs post infection



72hrs post infection

- Transmission electron micrographs show *C. burnetii* infection within a clearly defined CCV (white arrows)
- By 72hrs post infection the CCV has expanded to occupy the entire cell
- Currently working on developing *in vitro* assays paired with fluorescence microscopy to determine the presence of CCV markers (e.g. LC3, Lamp-1)

Transcriptome of *C. burnetii* isolated from infected *G. mellonella*

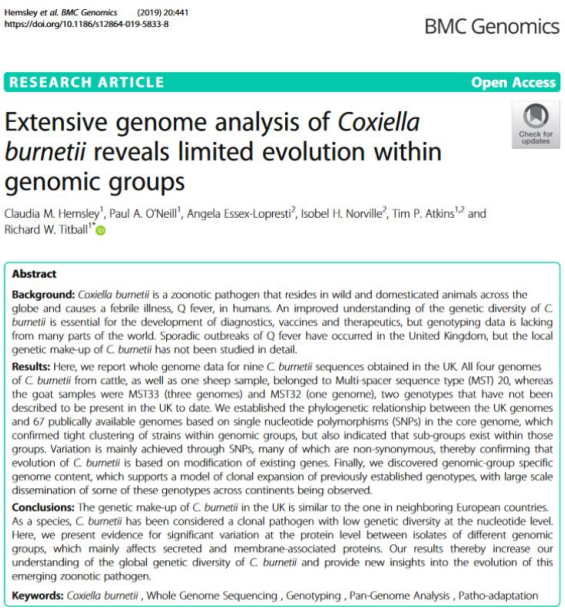


- The T4SS structural components are not upregulated (but they are expressed)
- Some effectors are upregulated, some downregulated

Q-fever

- If it is important why has it been neglected?
  - Until recently it was not to culture *C. burnetii* axenically
  - Isolation of *C. burnetii* from tissue samples is very challenging
  - Until recently there were no genetic tools
  - There is no good small animal model of infection

Genetic diversity of *C. burnetii* ?



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BMC Genomics

RESEARCH ARTICLE Open Access

Extensive genome analysis of *Coxiella burnetii* reveals limited evolution within genomic groups

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Abstract

Background: *Coxiella burnetii* is a zoonotic pathogen that resides in wild and domesticated animals across the globe and causes a febrile illness, Q fever, in humans. An improved understanding of the genetic diversity of *C. burnetii* is essential for the development of diagnostics, vaccines and therapeutics, but genotyping data is lacking from many parts of the world. Sporadic outbreaks of Q fever have occurred in the United Kingdom, but the local genetic make-up of *C. burnetii* has not been studied in detail.

Results: Here, we report whole genome data for nine *C. burnetii* sequences obtained in the UK. All four genomes of *C. burnetii* from cattle, as well as one sheep sample, belonged to Multi-spacer sequence type (MST) 20, whereas the goat samples were MST33 (three genomes) and MST32 (one genome), two genotypes that have not been described to be present in the UK to date. We established the phylogenetic relationship between the UK genomes and 67 publically available genomes based on single nucleotide polymorphisms (SNPs) in the core genome, which confirmed tight clustering of strains within genomic groups, but also indicated that sub-groups exist within those groups. Variation is mainly achieved through SNPs, many of which are non-synonymous, thereby confirming that evolution of *C. burnetii* is based on modification of existing genes. Finally, we discovered genomic-group specific genome content, which supports a model of clonal expansion of previously established genotypes, with large scale dissemination of some of these genotypes across continents being observed.

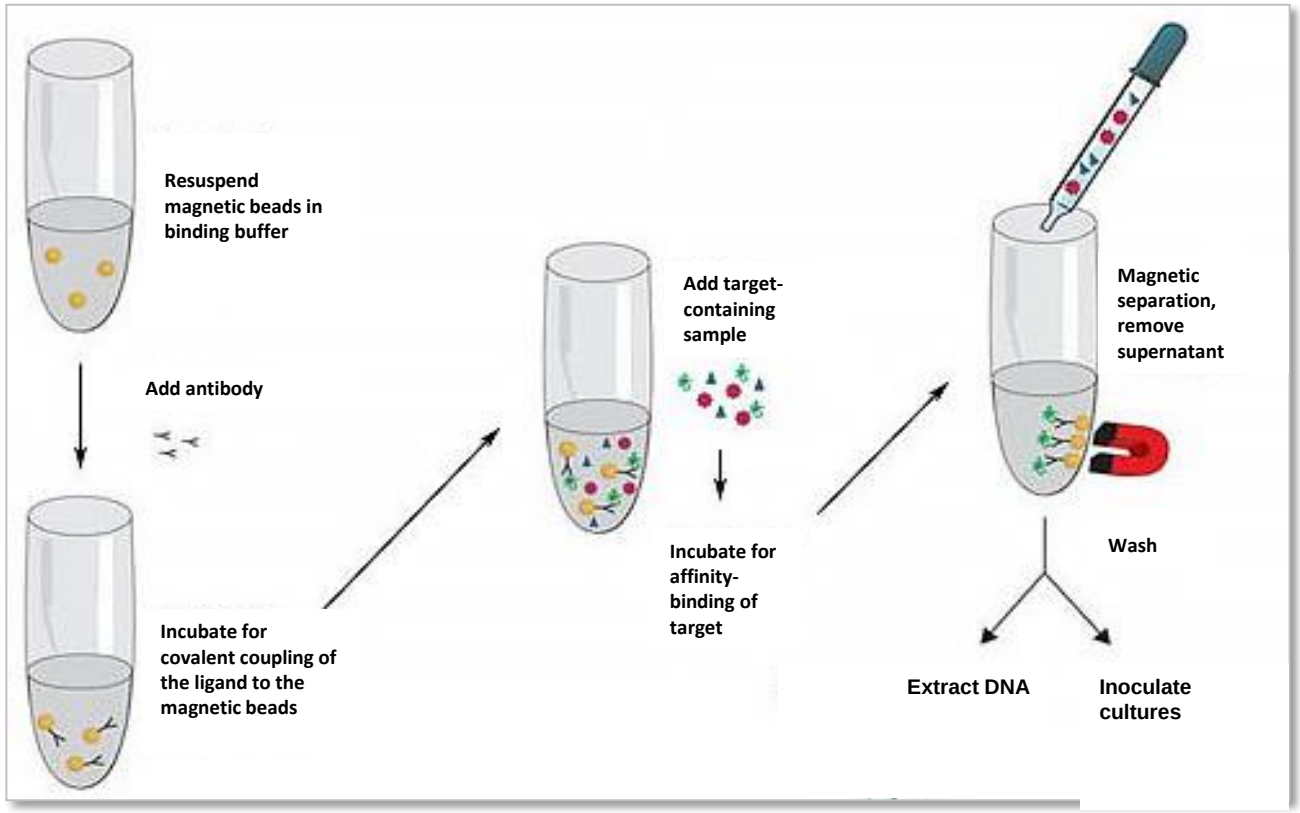
Conclusions: The genetic make-up of *C. burnetii* in the UK is similar to the one in neighboring European countries. As a species, *C. burnetii* has been considered a clonal pathogen with low genetic diversity at the nucleotide level. Here, we present evidence for significant variation at the protein level between isolates of different genomic groups, which mainly affects secreted and membrane-associated proteins. Our results thereby increase our understanding of the global genetic diversity of *C. burnetii* and provide new insights into the evolution of this emerging zoonotic pathogen.

Keywords: *Coxiella burnetii* , Whole Genome Sequencing , Genotyping , Pan-Genome Analysis , Patho-adaptation

- Partial or complete genomes deposited at GenBank
- Sequencing genomes of UK isolates
  - Human heart valve (1)
  - Cow placenta (4)
  - Sheep placenta (1)
  - Goat placenta (4)

Sequencing genomes of UK isolates

Immunoaffinity capture of *C. burnetii*

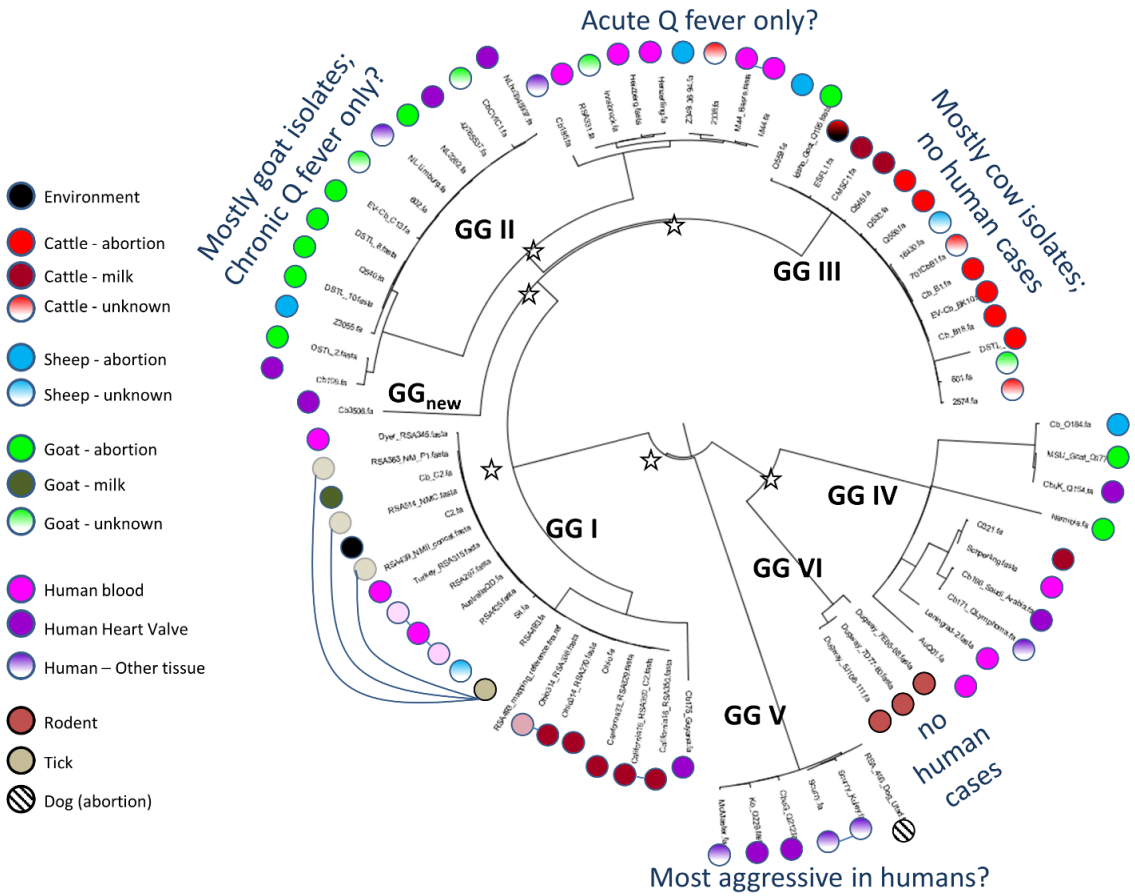


Schematic for immunoaffinity method. *Coxiella burnetii* Lane antibody was coupled to magnetic beads using the Dynabeads® Antibody Coupling Kit (Novex; Life Technologies)

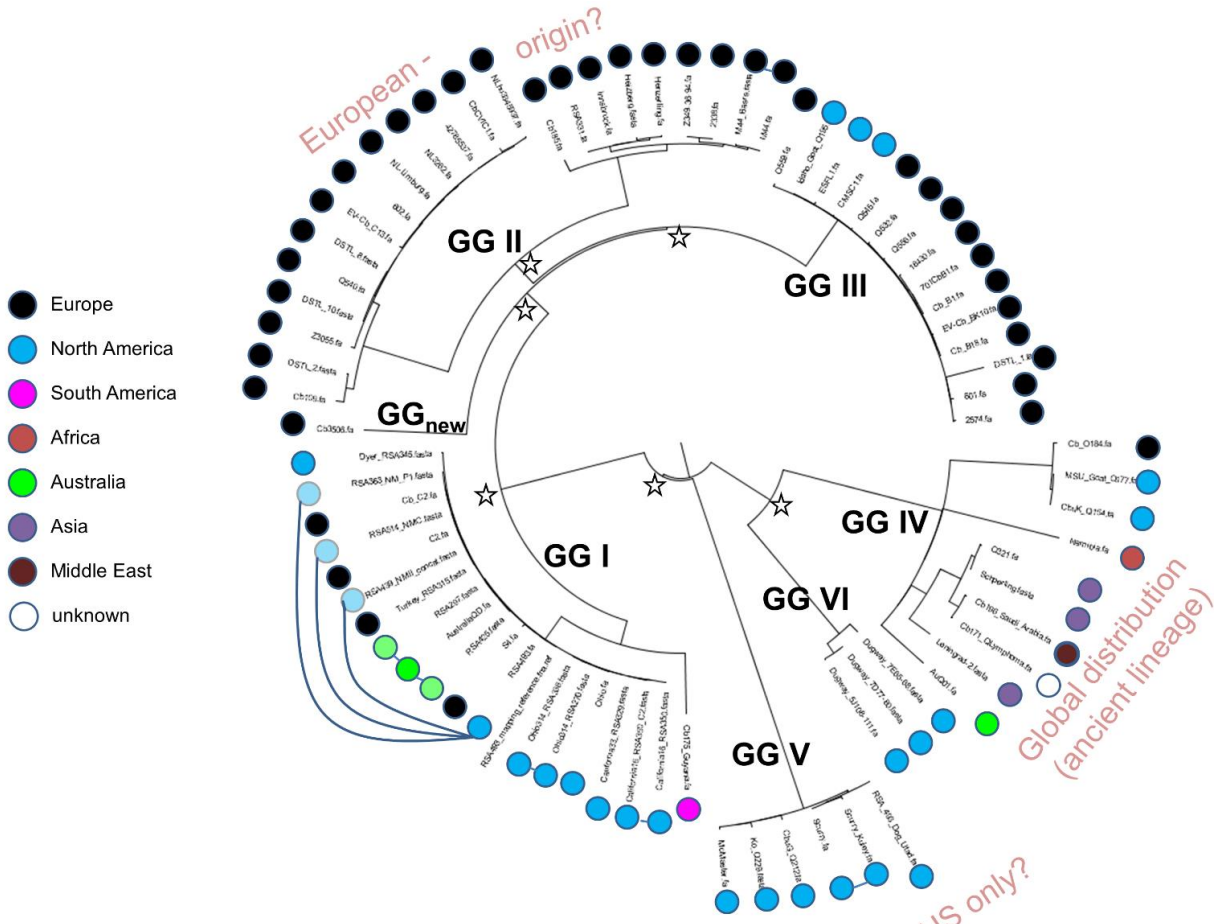
Core genomes of 76 *C. burnetii* isolates

Harvest
Suite
Tools

Lineage; association with source and human disease



Lineage; association with continent



Approaches to a vaccine

- Develop a sub-unit vaccine which could be used in both livestock and in humans

- Develop a for use in livestock, which may reduce exposure of humans to the bacteria

Q-fever vaccines in livestock

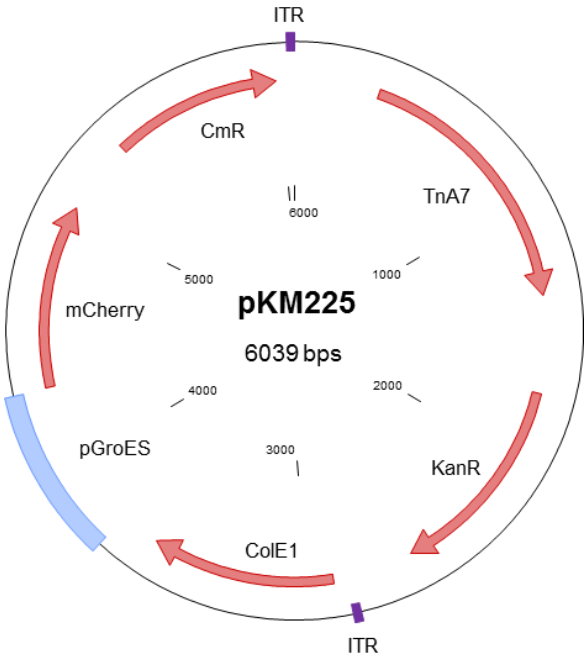
- There are a number of killed whole cell vaccines used in animals
  - 2 initial doses and boosters every 9-12 months
  - They are reactogenic (Schulze *et al*. 206;
  - They can reduce shedding from cattle in milk (Pinero *et al*., 2014; Taurel *et al*., 2014) or from goats (Muleme *et al*., 2017) or from sheep (Eibach *et al*.,2013)
  - They can result in a marked improvement in herd health (Lehner *et al*., 2017)

- There is an urgent need for an effective single dose vaccine for use in livestock

Live attenuated vaccine

- Need to be able to make and screen mutants
- Need a robust and reliable infection model

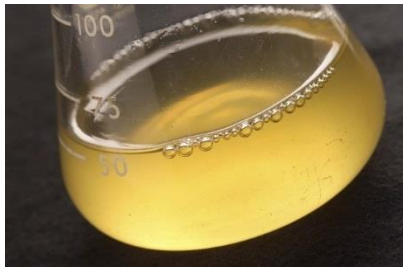
Himar1 transposon mutagenesis in *C. burnetii*



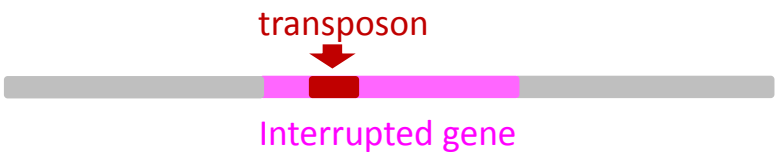
- *Himar1* Mariner family transposon
- Isolated from the horn fly *Haematobia irritans*
- Inserts randomly at TA sites
- Class II ‘’cut and paste’’ transposition mechanism
  - *Himar1* transposase recognises ITRs, facilitating transposon insertion
  - Transposase encoding gene encoded outside of ITRs = formation of a mutant with a single transposon insertion
  - AMR gene within ITRs, allows for selection of successful mutants

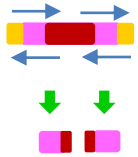
Sequencing allows a global population of mutants to be mapped

Pool of *C. burnetii* transposon mutants



Map mutant pool by sequencing DNA flanking each transposon





PCR amplification and sequencing

Location and abundance

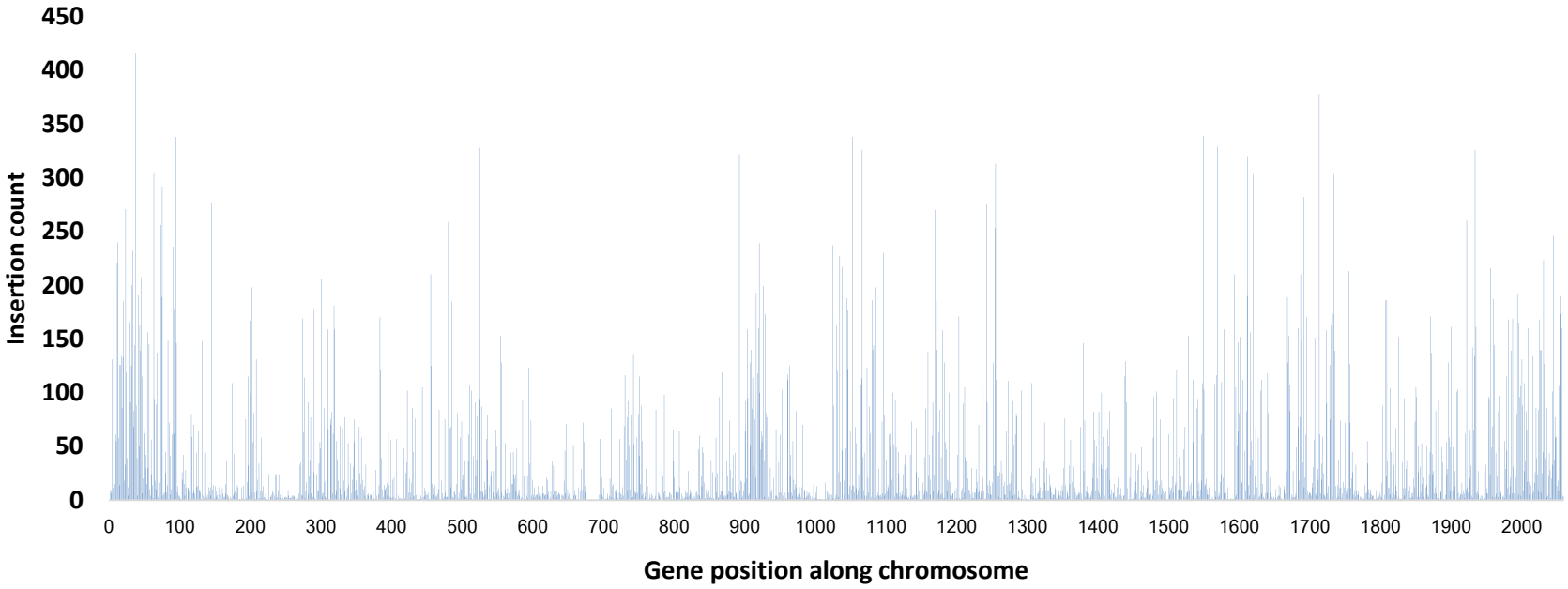
Transposon mutagenesis

Detect and remove transposon tags

Map reads to genome to identify insertion site

Calculate insertion index normalised to gene length

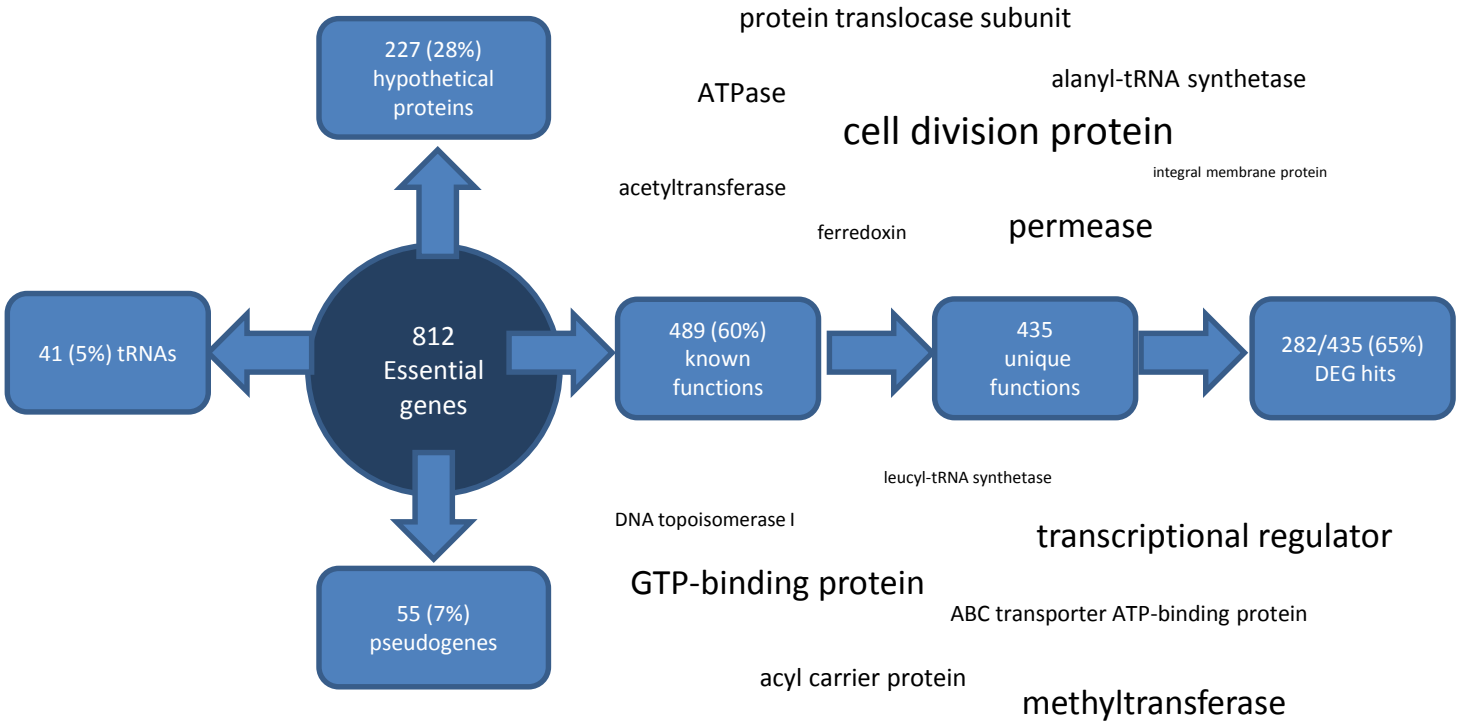
Total Reads	20,826,953
Reads Matched	19,771,986
% Matched	94.93
Reads Mapped	18,617.42
% Mapped	94.16
Unique Insertion Sites	89,122



Some genes are not interrupted by transposons

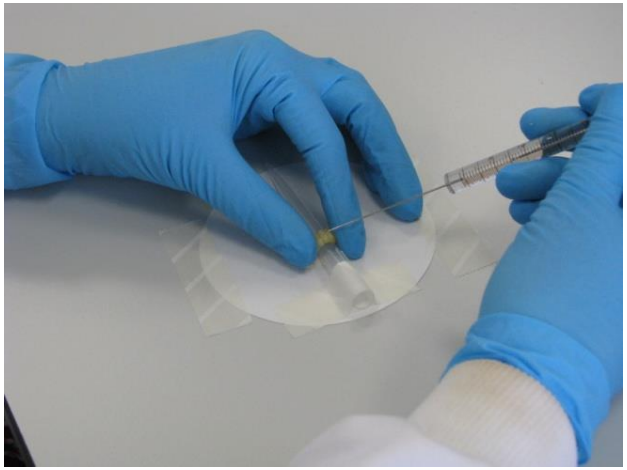
- Mutation renders the bacterial cell non-viable
- “Essential” genes

Essential genes in *C. burnetii*



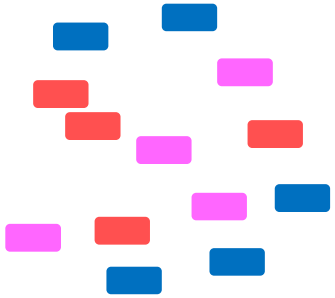
G. mellonella as a TRADIS infection model

- Challenge with transposon library
- Identify mutants that are eliminated
- These potential virulence genes



C. burnetii genes required for growth *in vivo*

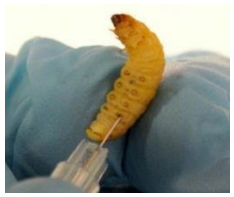
Pool of *C. burnetii* transposon mutants



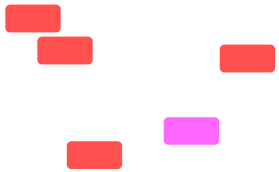
INPUT POOL

ESSENTIAL GENE

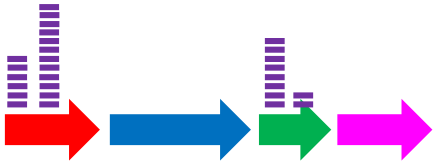
Sequence DNA flanking the transposons



Recovered pool from infected *G. mellonella*



OUTPUT POOL



VIRULENCE GENE

Summary

- *Coxiella burnetii* is an under-estimated cause of animal and human disease
- Some genomic groups are more likely to be associated with human disease
- A One Health approach could enable disease in animals and humans to be controlled
- Global mutagenesis is being used to identify a live attenuated vaccine candidate

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