Lyme disease: phages for diagnosis and treatment

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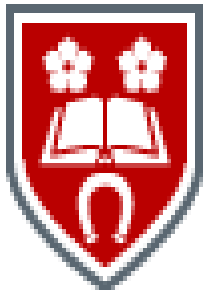

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ILADS 20th Annual Conference
Chronic Disease = Chronic Infections?
The Westin Copley Place Hotel
Boston, Massachusetts, Oct 31-Nov 3, 2019

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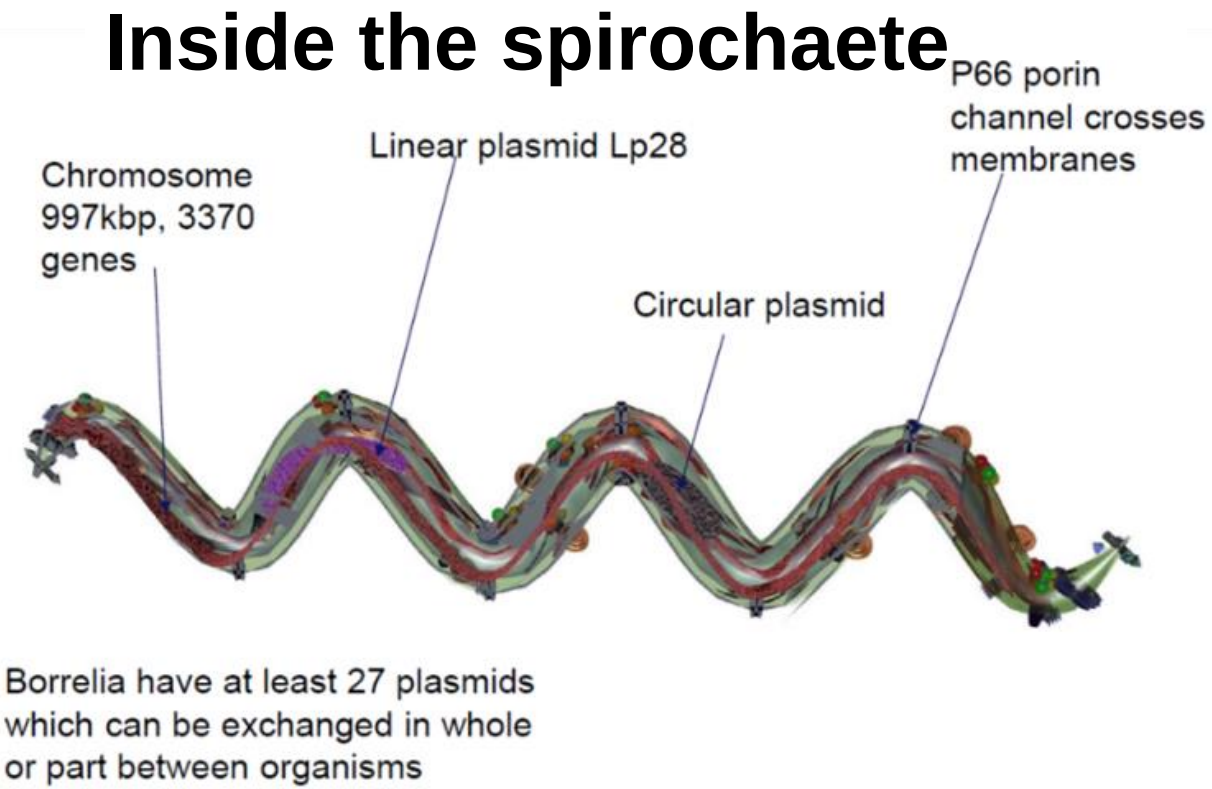
Talk Outline

- **What are the current problems in diagnosis (and maybe treatment)of LD?**
- **How phages can help?**
  - **Treatments**
  - **Diagnostics(phages targeting *Borrelia* (and other hard to diagnose**

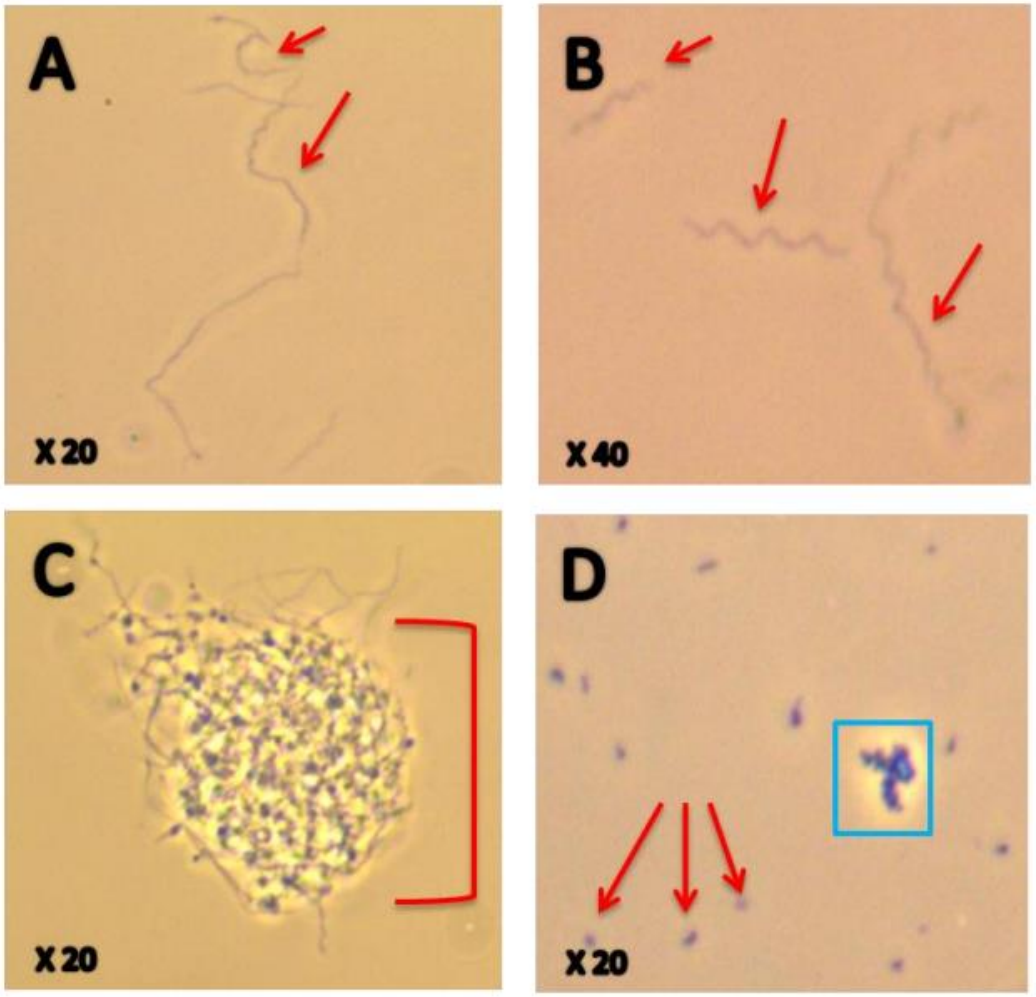
Borrelia strains have a unique genetic makeup that contributes to antibiotics' lack of efficacy

- a main chromosome (911 kb for the type strain B31), and 20 or more smaller plasmids ranging from 5-50 kb
- Cp32 plasmid family of *Borrelia burgdorferi* has been demonstrated to be a bacteriophage



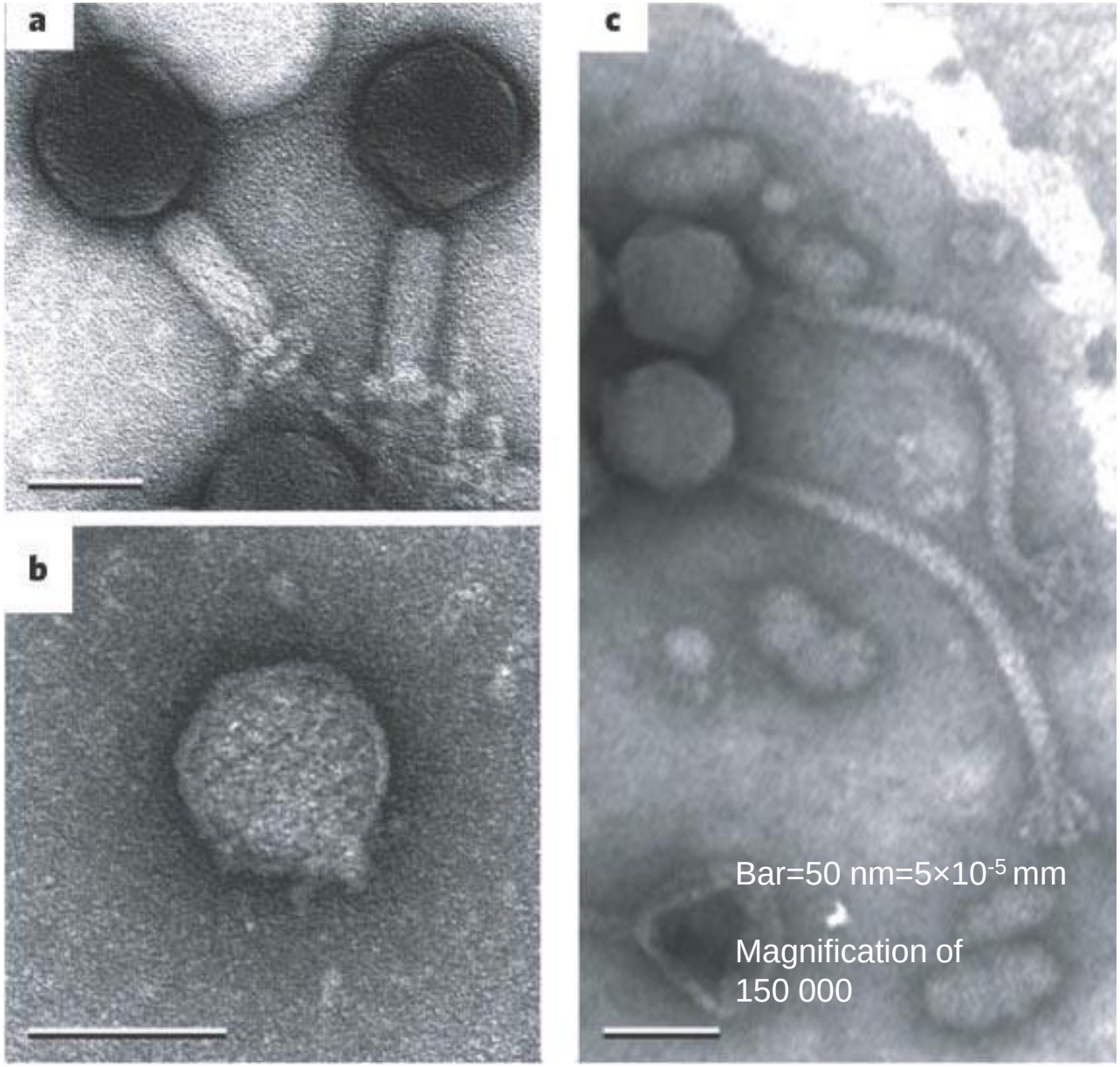
Borrelia (resistance/surviving)forms

antibiotics' lack of efficacy

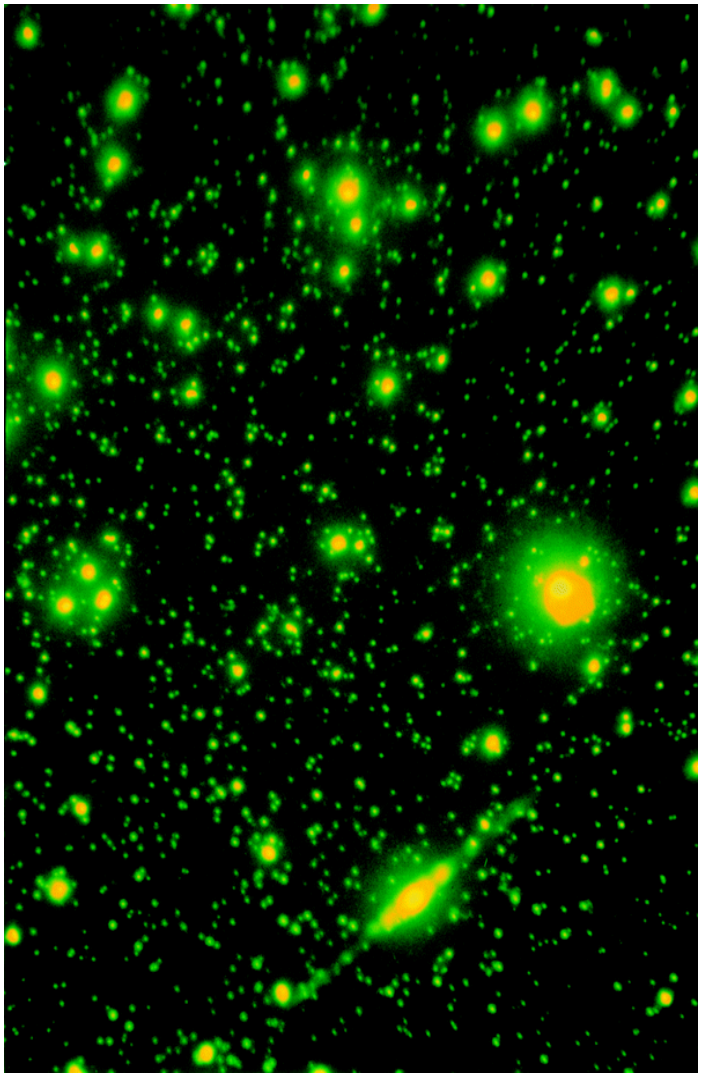


Diagnostics	Qualities and defects
Antibody-based	• Give indirect evidence • Low sensitivity (early stages) • Can't distinguish active and non-active *Borrelia* presence • *Difficult identification of Borrelia* sub-types
Bacterial DNA-based	• Direct evidence of *Borrelia* presence • Low sensitivity • Can't distinguish live and dead *Borrelia* • Might be able to tell different *Borrelia* sub-types
Lymphocyte transformation test	• Provide indirect evidence • Variable sensitivity that depends of immune system status and interfering treatments. False positive if time of incubation >24hours not reflecting real situation • Can only detect Lymphocytes that have been in contact with *Borrelia* within 45±15 days, thus limited in application

How phages look like?



Phages are viruses that infect bacteria, and they are everywhere



Fluorescence imaging of marine viruses (Fuhrman J.A. 1999)

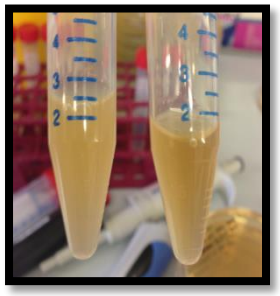
50 million (5×10^7) viruses per milliliter of seawater

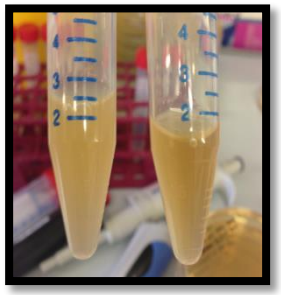
Estimated number of phages:
The open ocean: 1.2×10^{29}
The soil: 2.6×10^{29}
The ocean sediments: 3.5×10^{30}
The terrestrial sub-surfaces: $0.25–2.5 \times 10^{30}$

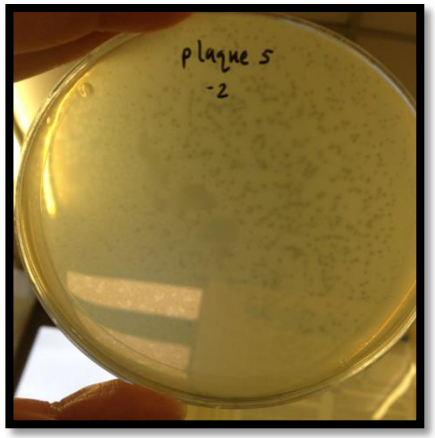
- There are 10/20 times more phages than bacteria

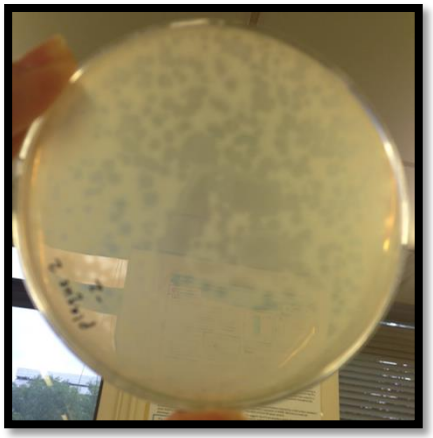
Phage isolation

+

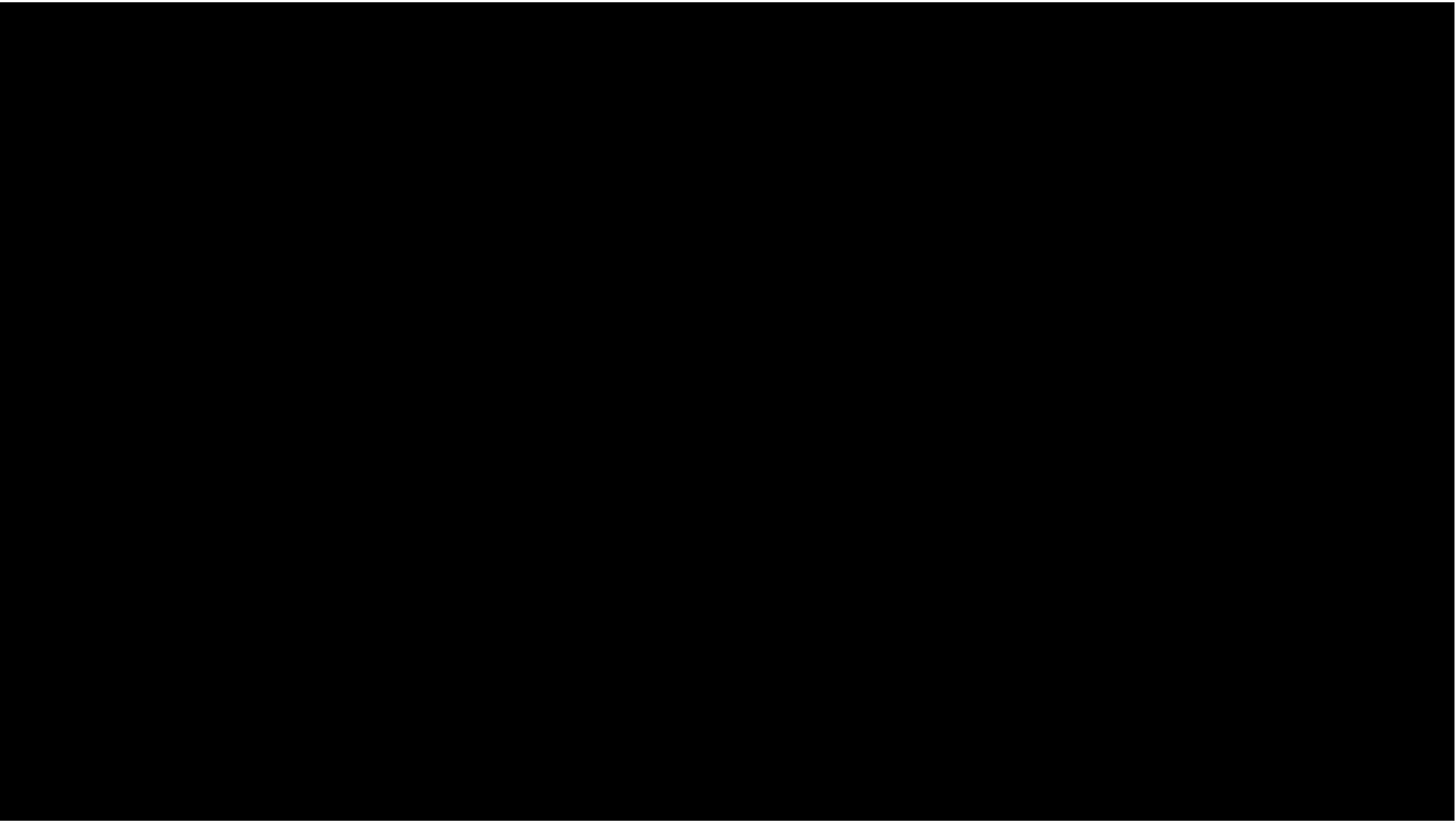


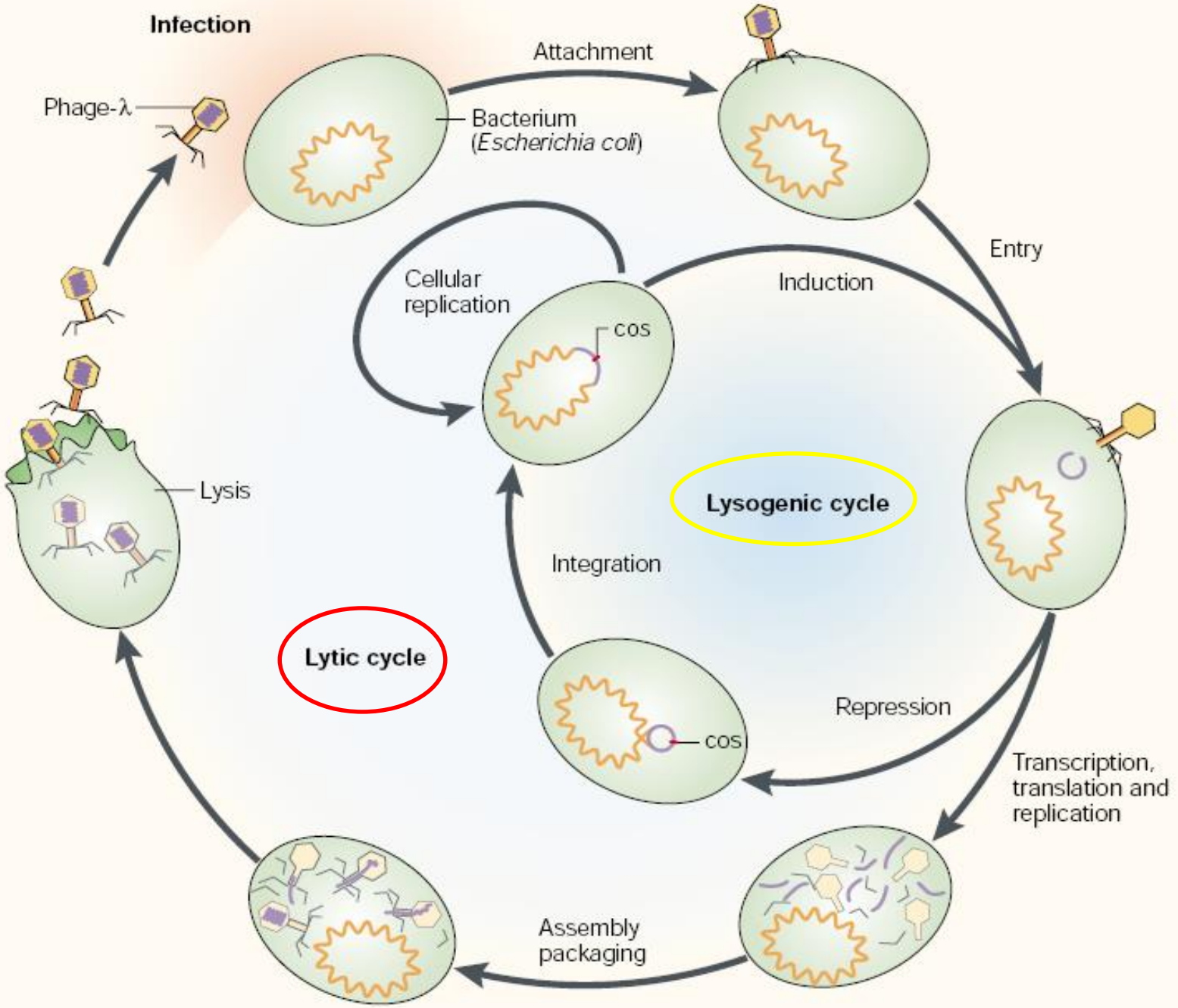






Phage life cycle



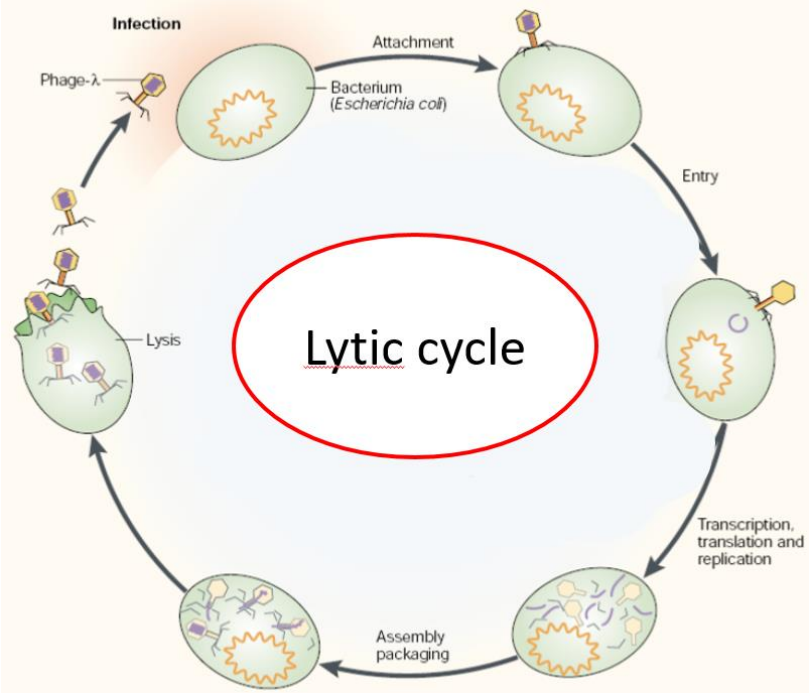

Infection
Phage-λ
Attachment
Bacterium
(Escherichia coli)
Entry
Induction
Cellular
replication
cos
Lysogenic cycle
Integration
Lysis
Lytic cycle
cos
Repression
Transcription,
translation and
replication
Assembly
packaging

Virulent Phages (1)

The virulent phage, sometimes also called "lytic", infects its target and starts its reproduction immediately by mobilizing the resources of the host in its favor.

The viral genes are then expressed in a very precise and closely regulated order. The first proteins produced, the early proteins, are responsible for phage multiplication and, in many cases, interrupt the synthesis of cellular proteins.

Some virulent phages are even capable of degrading the host genome and monopolizing cellular metabolism for their own reproduction.

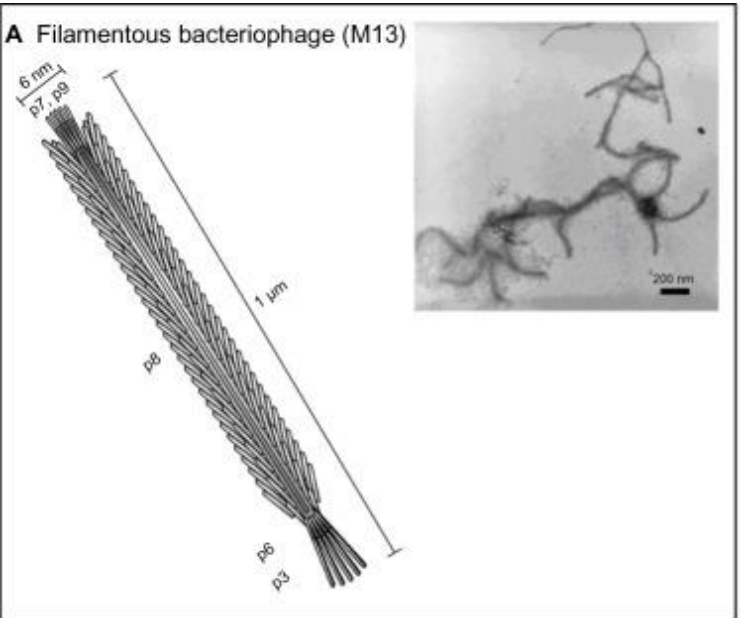


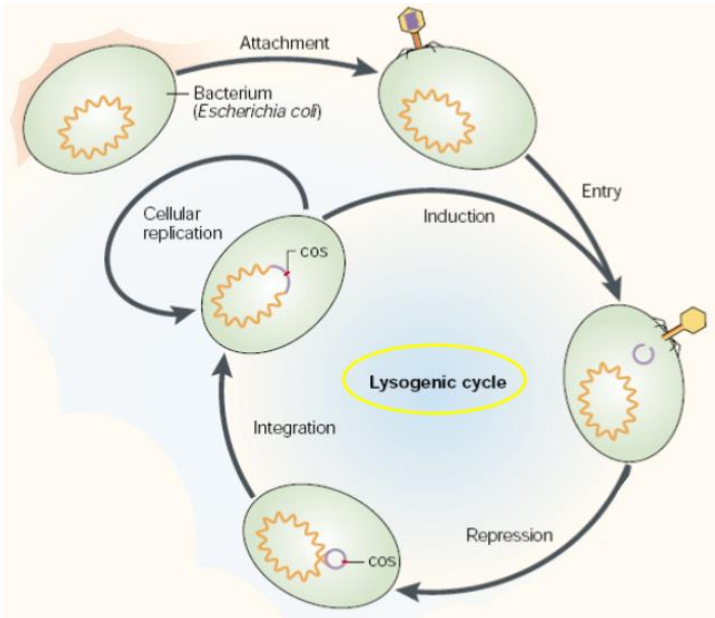
Filamentous Phages

When nucleic acid (generally single-stranded) is injected into the target cell, the capsid proteins are inserted into the membrane.
Once inside the host, the genome is abundantly replicated and the genes necessary for the synthesis of structural proteins are expressed.

Proteins will in turn enter the cytoplasmic membrane and, together with the structural proteins inserted in the membrane during infection, will serve to form the new capsids.
The phages are then secreted through the cell wall via a channel formed of three viral protein species according to a process consuming ATP.

Unlike other types of phage, filamentous phages do not kill their host, but are released as they replicate. This interesting characteristic makes it the tools of choice in molecular biology



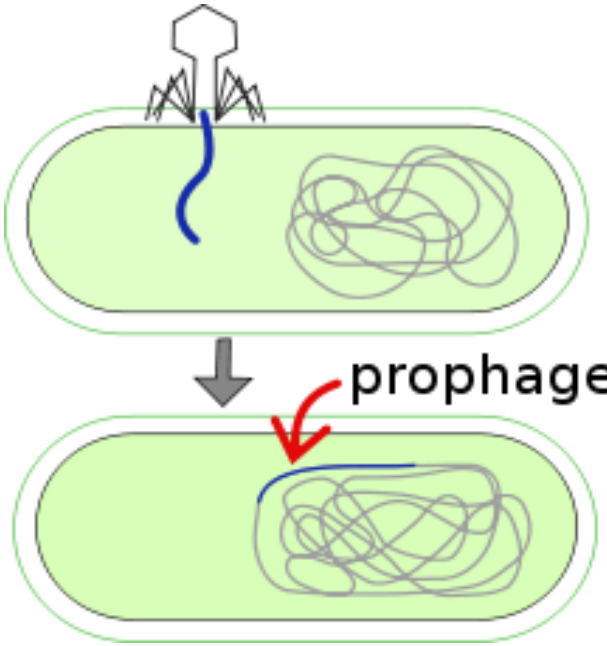


Prophages

In most cases, viral DNA integrates physically into the host genome and is copied with the entire genome as the cells divide.

This state may persist for several generations and the host cell is then said to be lysogenic.

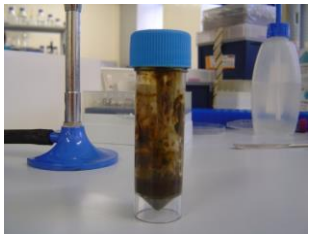
The quiescent state is maintained by a repressor of the lytic functions. Its role is to ensure the stability of the prophage state and at the same time to enable it to enter the active phase rapidly when circumstances demand it.

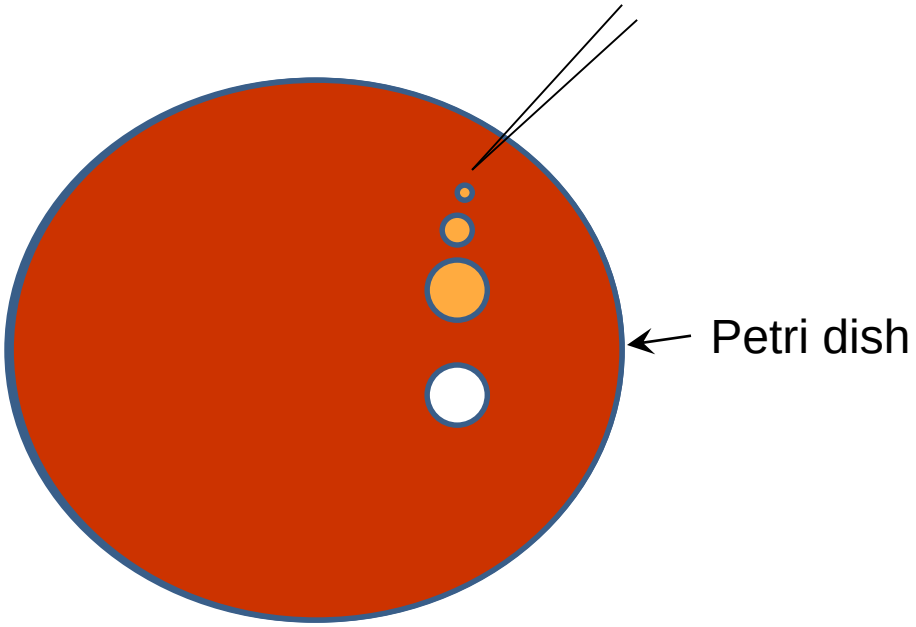


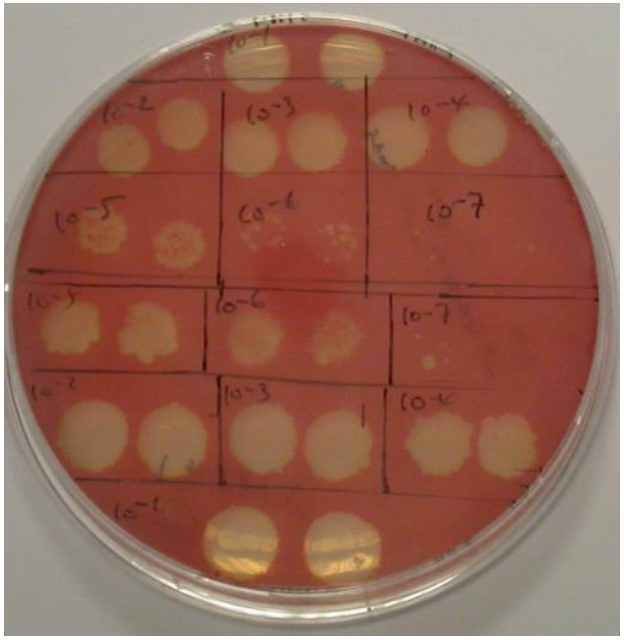
This is the case when the bacterium is exposed to a deficiency or stress damaging its integrity for example.

The prophage then comes out of its quiescent

Sampling: where there are bacteria, there are phages







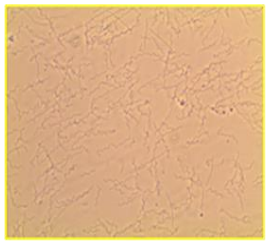
An example of ‘spot test’: the red background was a bacterial lawn, the clear spots were ‘phage drops’ with serial dilutions. Single ‘plaque’ can be seen

Three strategies for *Borrelia phages*

The hunt for *Borrelia* phages from wild ticks

Tick guts were subjected to Borrelia/phage enrichment. The enrichment samples are examined for *Borrelia* and phage presence

The hunt for *Borrelia* phages from *Borrelia*



Borrelia cultures were treated with low concentration of antibiotics to induce phages

After filtration, the filtrates were examined by transmission electron microscope (TEM)

To engineer phage proteins in killing *Borrelia*

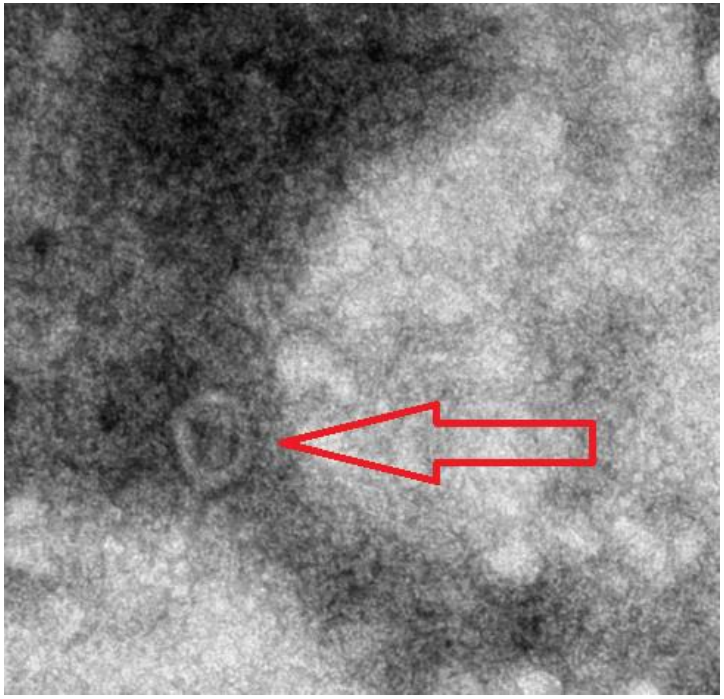
Overexpression and purification of phage-encoded enzymes (holins and endolysins)

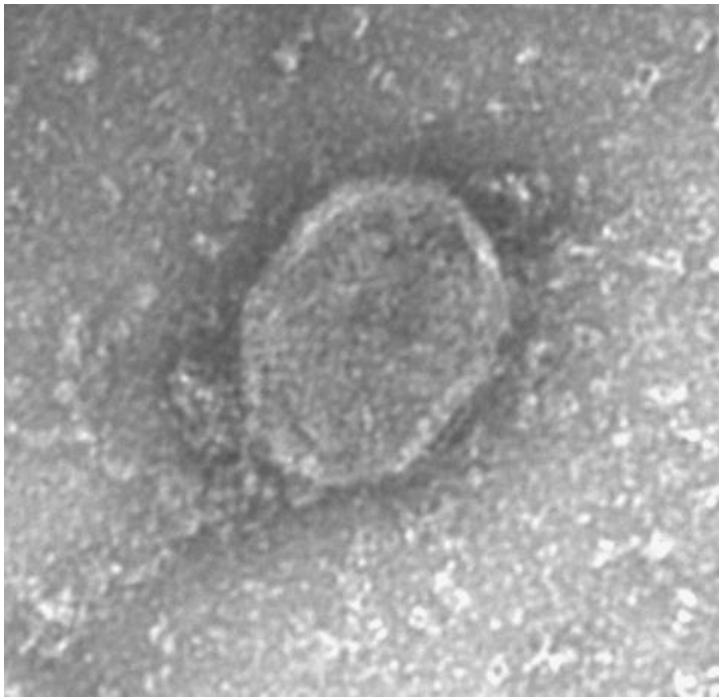
Holins and endolysins were bioinformatically identified from *Borrelia* genomes

Holins and endolysin were overexpressed in a yeast system. Purified proteins were tested against *Borrelia* strains

Our Research & Development

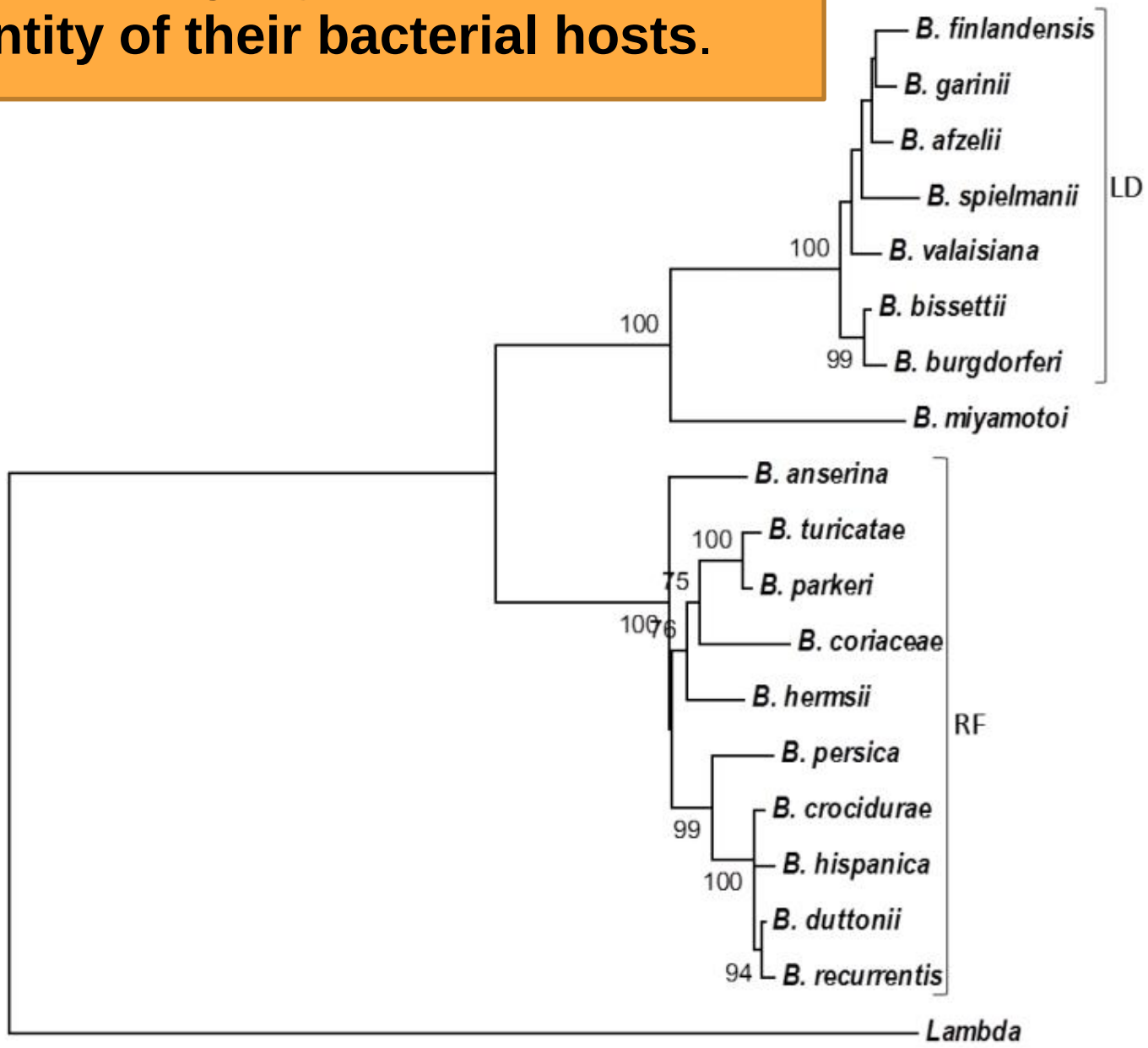
We were able to induce Temperate phages from Lyme *Borrelia* strains (table below).
We increased the phage concentration and phage





Phages can be seen under transmission electron microscope (TEM)

Phages residing within *Borrelia* strains are tightly correlated to the identity of their bacterial hosts.



Phage-based test design and calibration

RATIONALE: Phages are specific , circulate and are much more numerous than their targets

*Phelix Charity (chronic infections and bacteriophages research group) Department of Infection, Immunity, and Inflammation, **Uniiversity of Leicester***

OBJECTIVES

- **Determine the sensitivity and specificity of a phage-based PCR diagnostic method** in detecting the presence of Borrelia in blood
- **Validate a phage-based PCR diagnostic method** against the current antibody-based (ELISA + WB) and bacteria PCR-based Methods.

Phage-based PCR:

Several sets of PCRs specifically targeting the conserved regions of Borrelia phages residing inside Borrelia strains were applied to the DNA extracted from the whole blood and sera samples. These PCR primers include one set that targets all of the Borrelia burgdorferi s.l., and several other different sets of primers/probes that are specific for Borrelia afzelii, Borrelia garinii, and Borrelia miyamotoi. The PCR product is visualised based on a capillary gel system, and a Taqman qPCR system.

Method development: specificity

- PCR primers were designed targeting conserved regions within *Borrelia* phages.
- PCR was validated against all known bacteria using *in silico* PCR (http://insilico.ehu.eus/PCR/).
- 'Wet PCR' were performed against LD&RF *Borrelia* strains and the following bacteria in the lab, such as *Clostridium difficile*, *Burkholderia thailandensis*, *E. coli*, *Salmonella*, *Legionellae*, *and Haemophilia* strains None of these bacteria generated any PCR products.

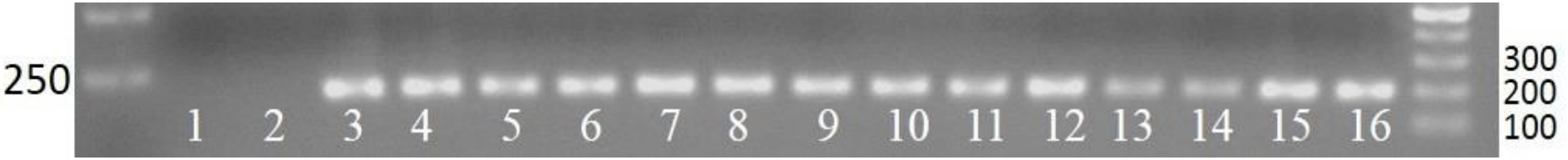


Fig. Phage PCR was carried out against different Lyme *Borrelia* strains.

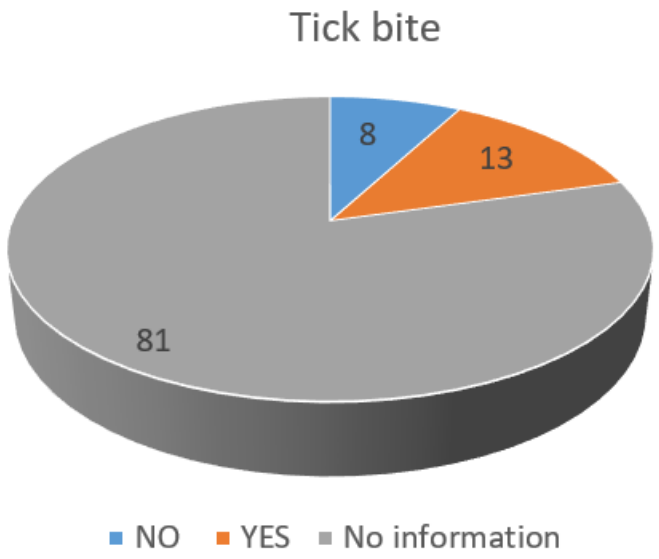
A single PCR product was generated from each DNA sample with the expected size and sequence. PCR was run in duplicate for each DNA template. Every two lanes represent one PCR as follows: 1, 2: negative control; 3, 4: *B. burgdorferi* B31; 5,6: *B. burgdorferi* VS185 P9; 7,8: *B. valaisiana* NE218; 9, 10: *B. afzelii* ACA1; 11, 12: *B. burgdorferi* UK filtered; 13, 14: *B. garinii* 190 P9; 15, 16: *B. burgdorferi* China23. The size of DNA ladders on both edges

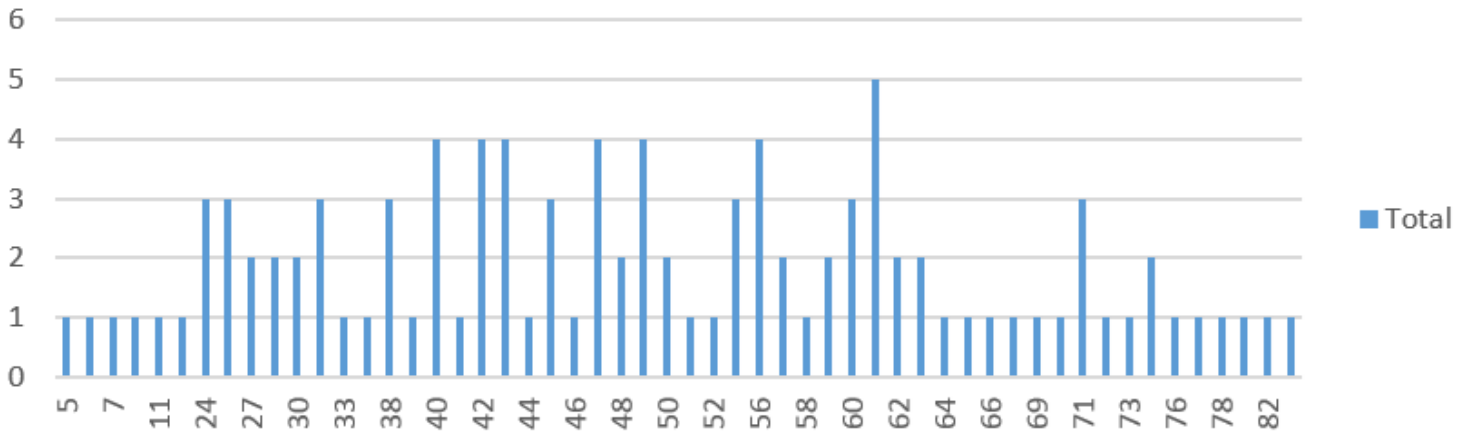
Method development: sensitivity

- The phage PCR and bacterial PCR were carried out against four *Borrelia burgdorferi* B31 culture, which has been diluted down to 10 *Borrelia*/ml.

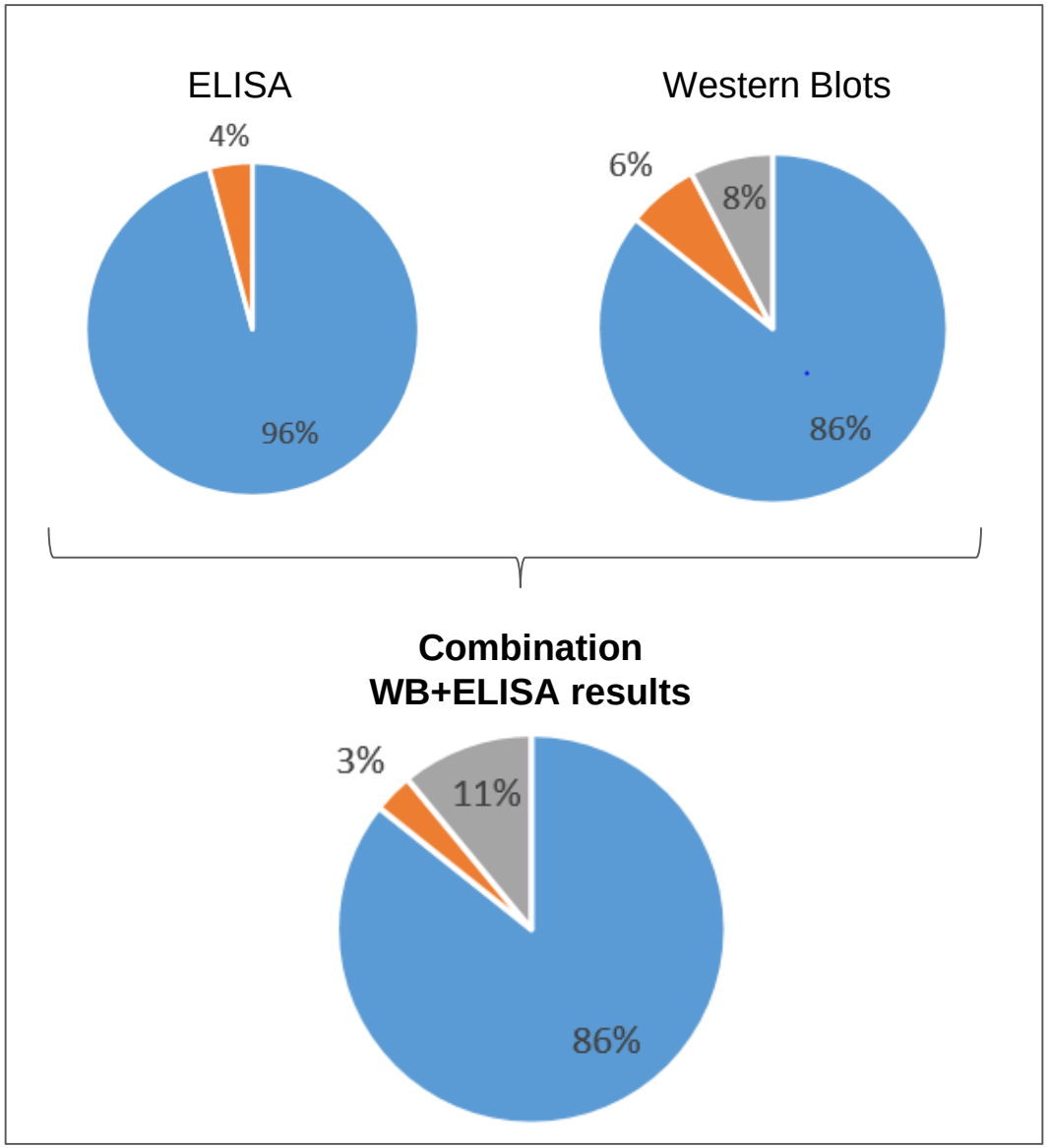
Fig. 2 Two strong PCR positives were observed from phage PCR (top panel), and one weak positive was observed from bacterial PCR. The DNA templates used in the PCR were extracted from diluted *Borrelia* cultures with a concentration

First results: Scope of study (102 people presenting Lyme disease





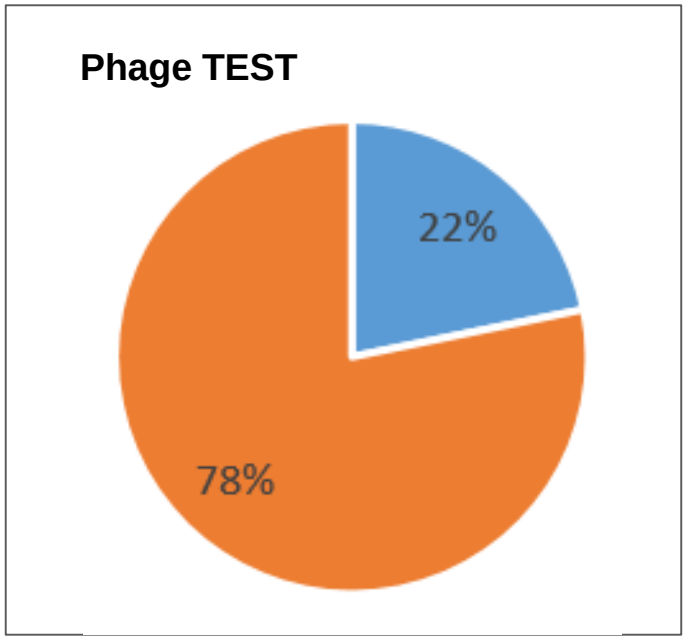
First results: Phage test versus serological IgG tests



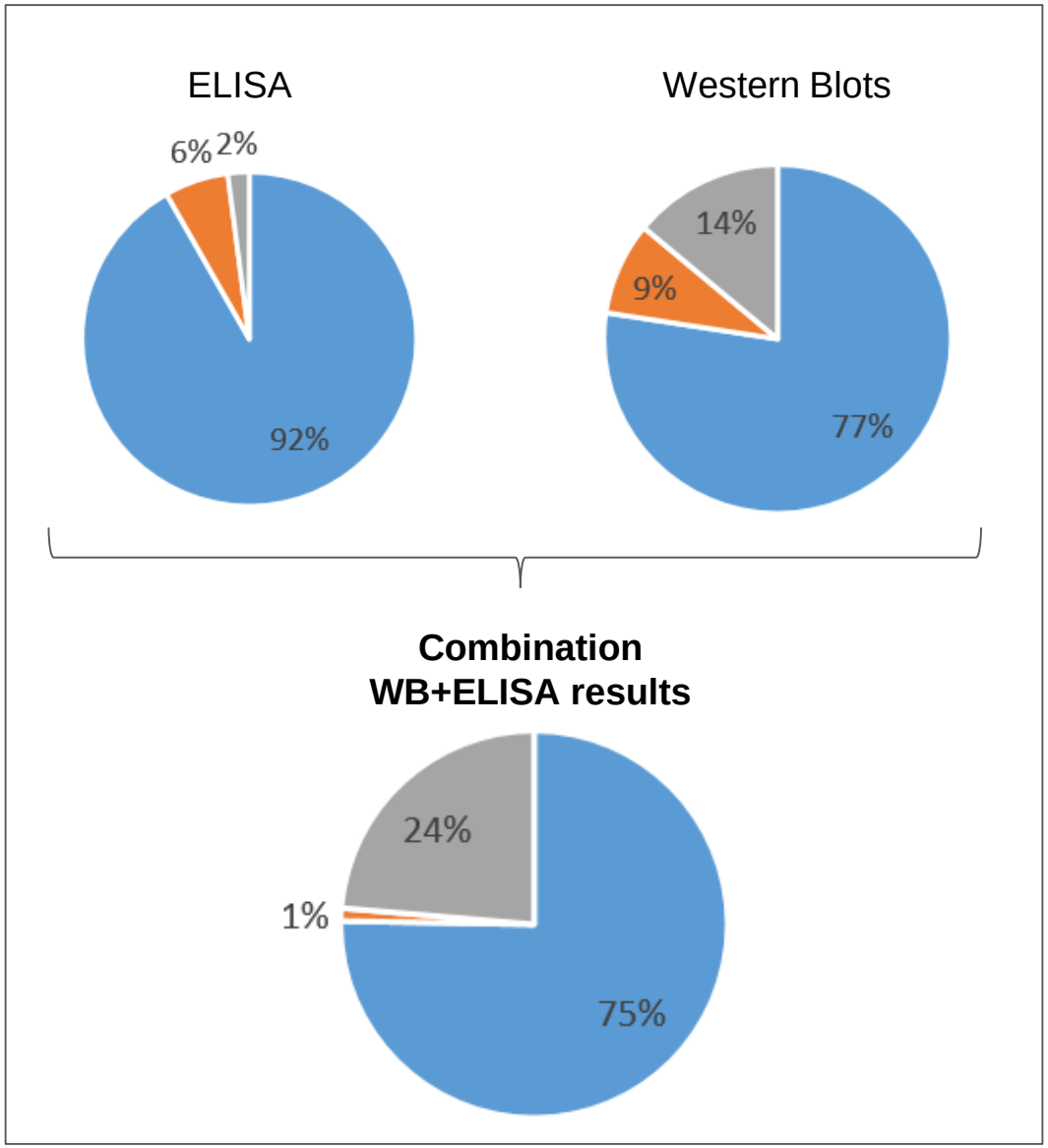
NEG POS DOUBT

Doubt means either:

- Negative with Blots
- Negative Border Line



First results: Phage test versus serological IgM tests



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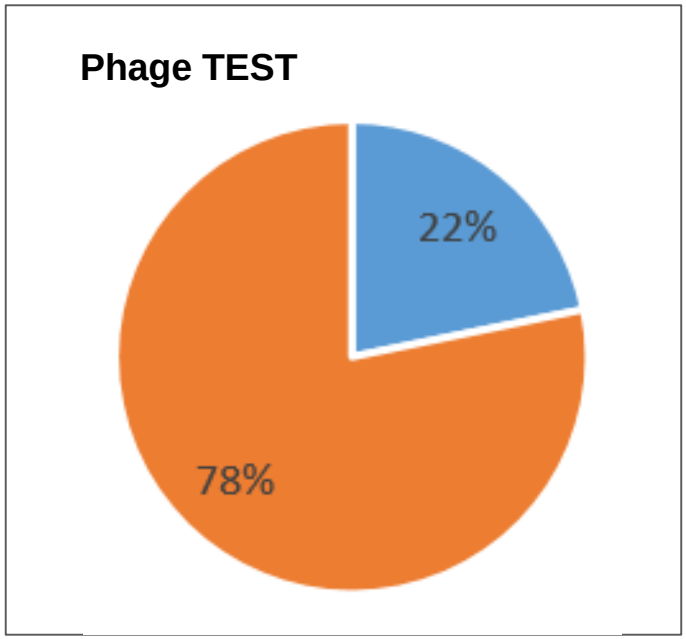


First results: activity detection

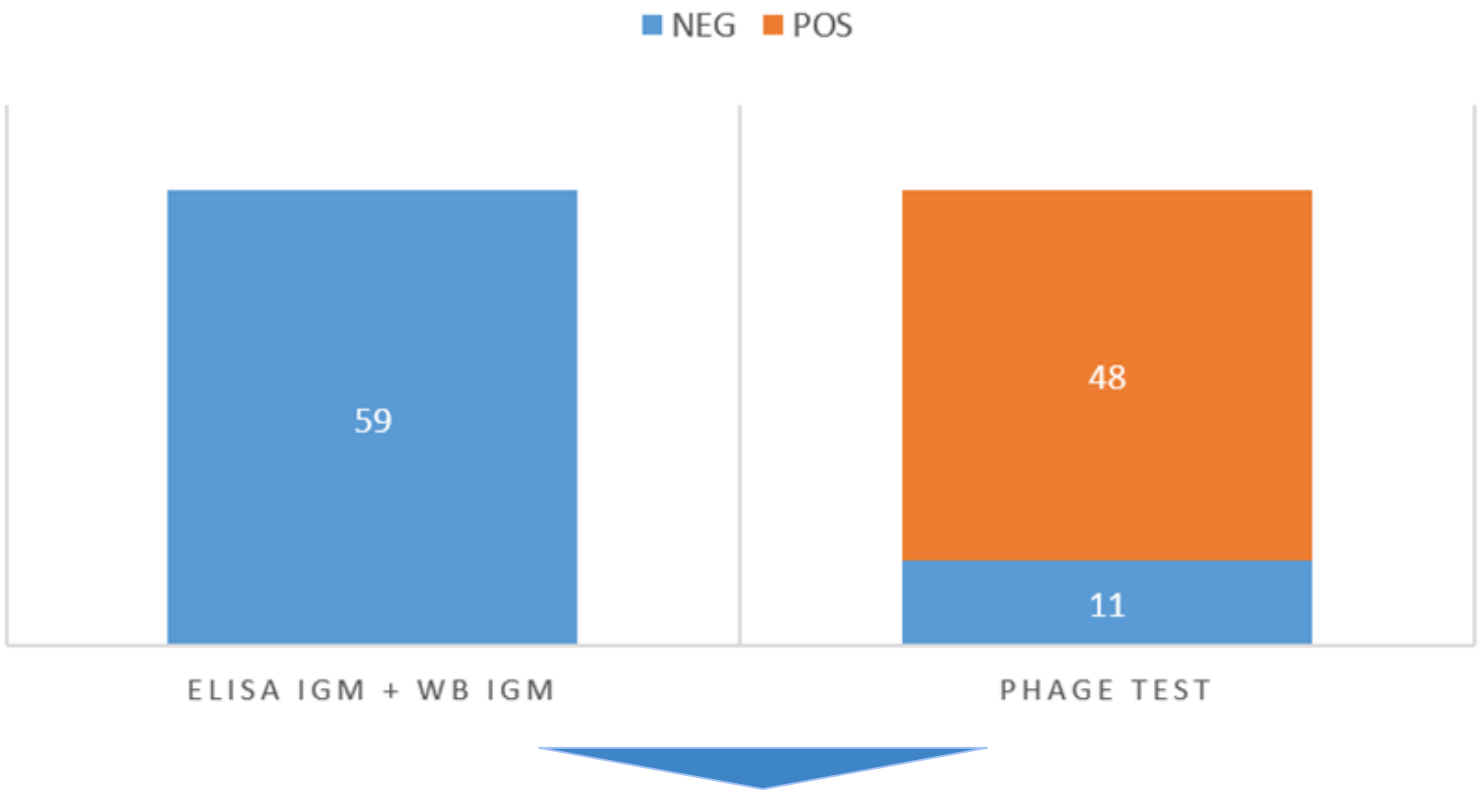
Focus on 3 patients with positive results in IgG :

	ELISA (IgG)	Western Blot (IgG)	Phage test
Patient 1	+	+	+
Patient 2	+	+	+
Patient 3	+	+	-

Phage test detects Borrelia active presence, while Elisa and Western Blots (IgG) only detect a former contact with the bacteria.

First results: false negative detection

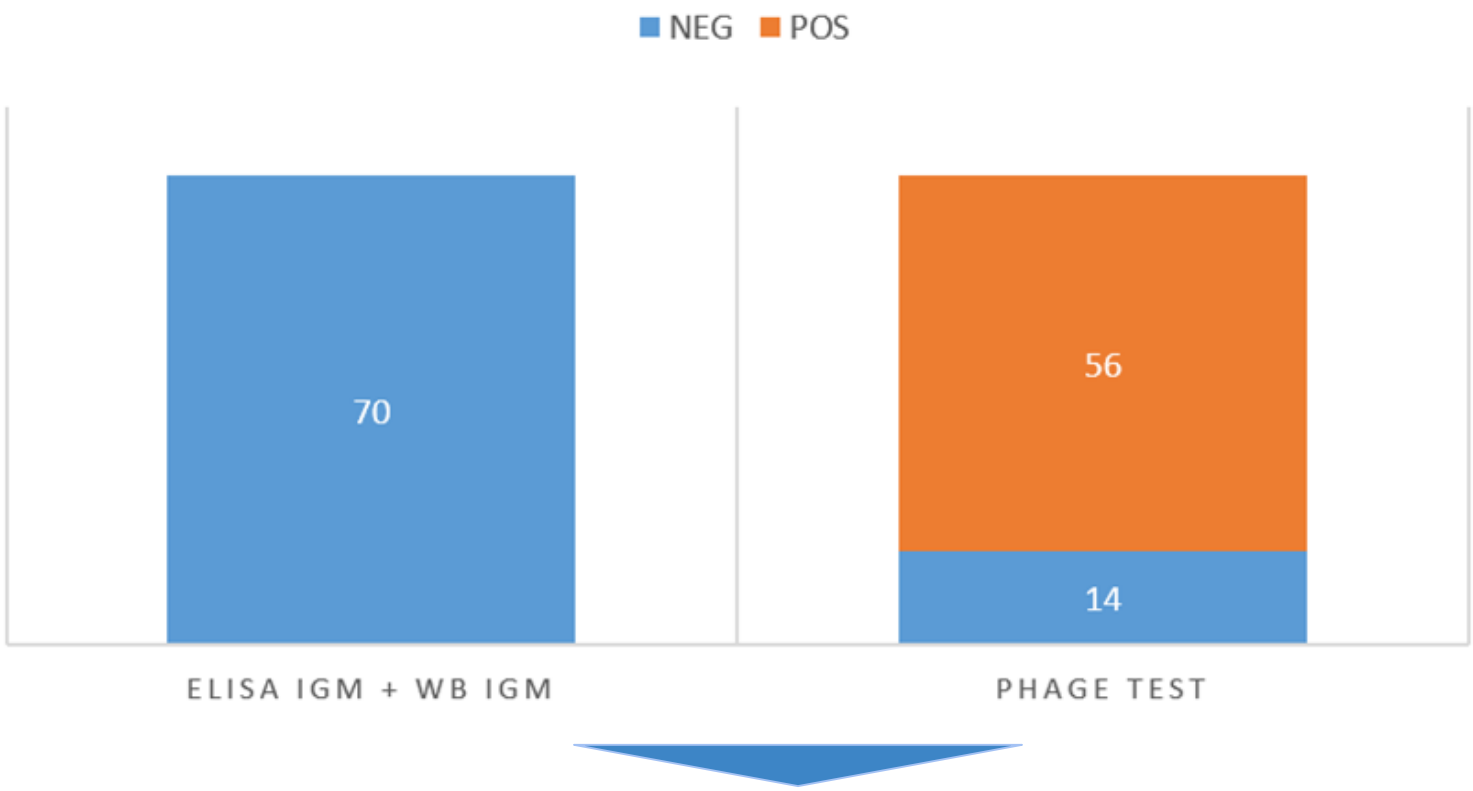
Focus on the patients with <u>negative results in both ELISA and WB IgG and IgM</u>:



Phage test detects the presence of active phages in a large number of patients having both negative ELISA and WB (IgG

First results: IgM false negative detection

Focus on the patients with <u>negative results in both ELISA and WB IgM</u> only:



Phage test detects the presence of active phages in a large number of patients having both negative ELISA IgM and WB IgM

Phage-based PCR test : validation

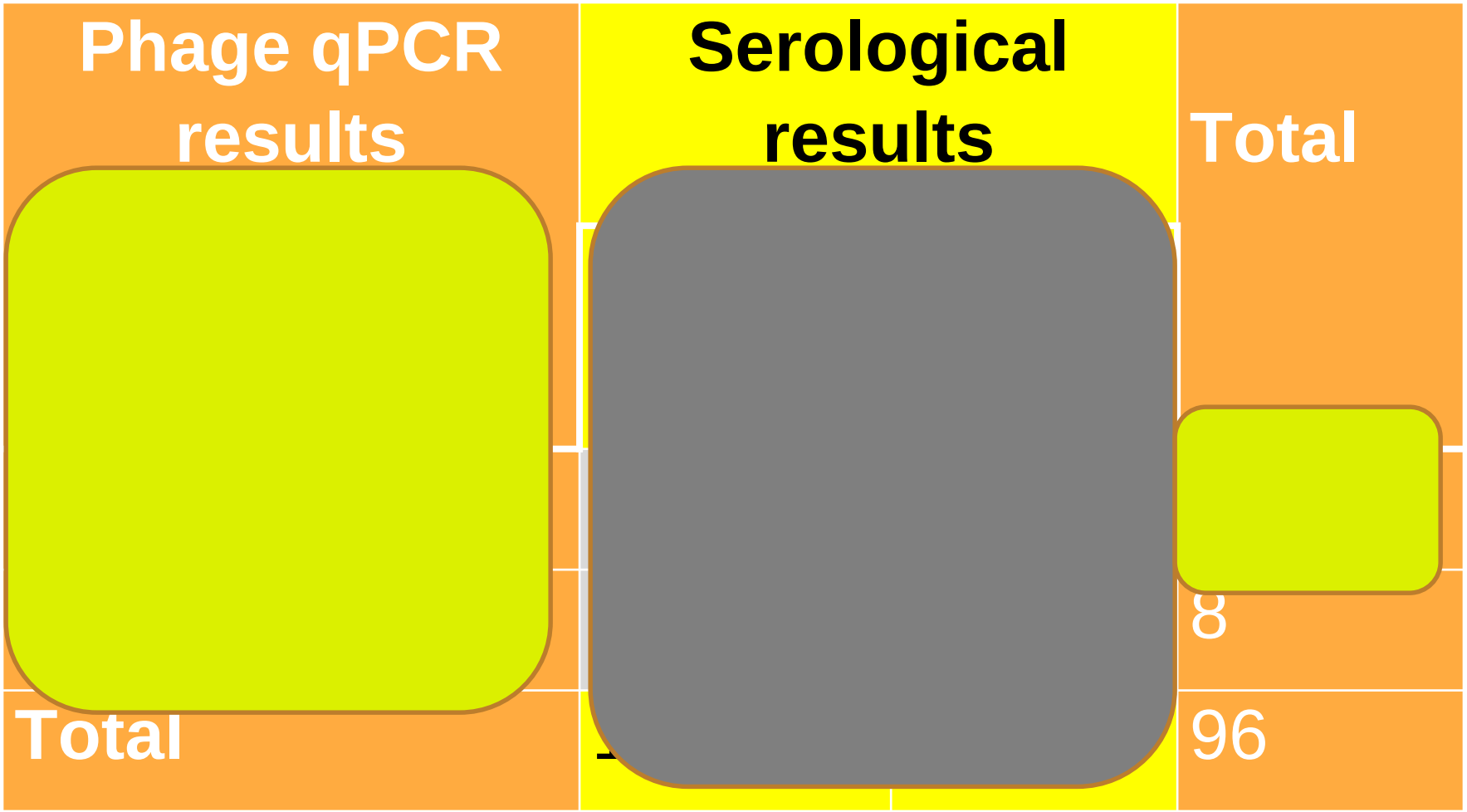
Its high sensitivity makes it able to detect the disease in the first weeks of infection as well as in the late stages

Phage-based PCR is an in vivo amplification system

It's high specificity permits to distinguish between the two tick-borne diseases and indicates the bacterial species involved

Performance of the qPCR in clinical samples

Table 1. Comparison of phage PCR and ELISA/WB Assays for



Phage qPCR: 88/96 × 100%= 92%

Serological assay: 15/96 × 100%= 16%

Performance of the qPCR in clinical samples

Table 2. Comparison of phage PCR and Bacterial PCR for detecting *Borrelia* in serum collected from 96 early stage patients who were clinically diagnosed as 'Lyme disease' by Dr Louis Teulieres.

Phage qPCR results	Bacterial qPCR		Total
	Positive	Negative	
Positive	33	55	88
Negative	0	8	8
Total	33	63	96

Phage qPCR: 92%

Bacterial qPCR: 34%

Performance of the qPCR against clinical samples

Table 3. Comparison of phage PCR and Bacterial PCR for detecting *Borrelia* in whole blood collected from 81 late patients who were clinically diagnosed as 'Lyme disease' by Dr Louis Teulieres.

Phage qPCR results	Bacterial qPCR		Total
	Positive	Negative	
Positive	31	40	71
Negative	0	10	10
Total	31	50	81

Phage qPCR: 88%

Bacterial qPCR: 38%

Performance of the qPCR in clinical samples

Table 4. Comparison of phage PCR and Bacterial PCR for detecting *Borrelia* in whole blood collected from 25 healthy volunteers who were organised by Dr Louis Teulieres.

Phage qPCR results	Bacterial qPCR		Total
	Positive	Negative	
Positive	3	9	12
Negative	0	13	13
Total	3	22	25

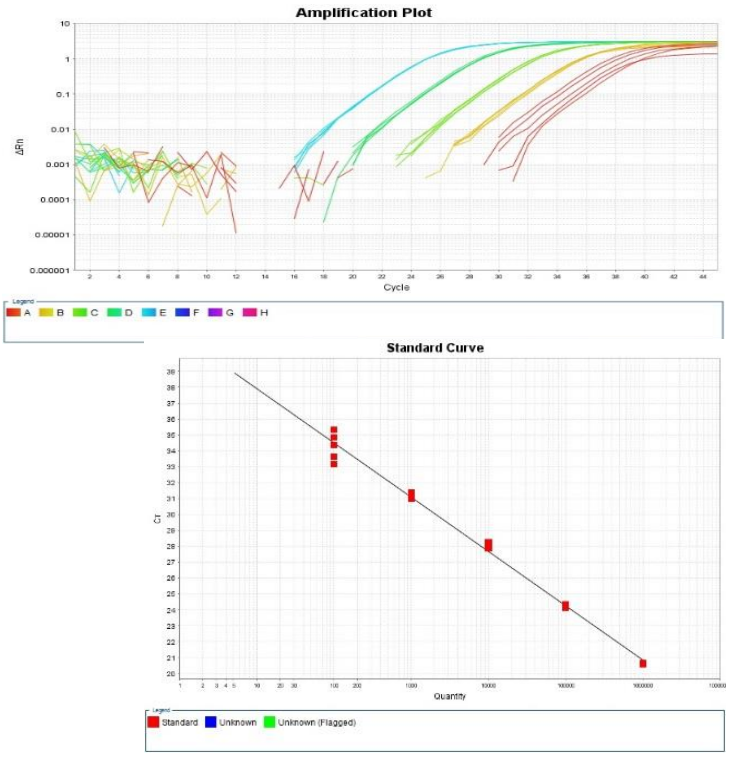
Phage qPCR: 48%

Bacterial qPCR: 12%

Detection of bacteriophage nucleic acids from whole blood and serum samples by a Taqman-based quantitative PCR with an internal control offers highly sensitive diagnosis of Lyme disease

- **Taqman primers and probes targeting phage genes + Taqman probe and primer set targeting an internal control are optimised/verified**

A standard curve from serial dilution of plasmid carrying phage gene is generated



- Determined the Limit of detection (LoD)

Copy number/PCR	Number of replicates	Number of PCR positive replicates (% of positive)
100	10	10 (100%)
80	10	10 (100%)
60	10	10 (100%)
40	10	10 (100%)
20	10	9 (90%)
10	10	7 (70 %)
5	10	2 (20%)
1	10	0

Summary of the phage-based test

- **Highly sensitive and specific.**
  - **The efficiency of the phage-based PCR was estimated by spiking human blood with known amount of *Borrelia* cultures. The lowest concentration that still generated a positive signal was 5 *Borrelia*/ml of blood.**
  - **Direct evidence of active *Borrelia* presence**
  - **Ability to distinguish different *Borrelia* sub-types)**
  - **Early stages anad late stages diagnostics .Treatments and medicines monitoring**

IN VIVO AMPLIFICATION SYSTEM

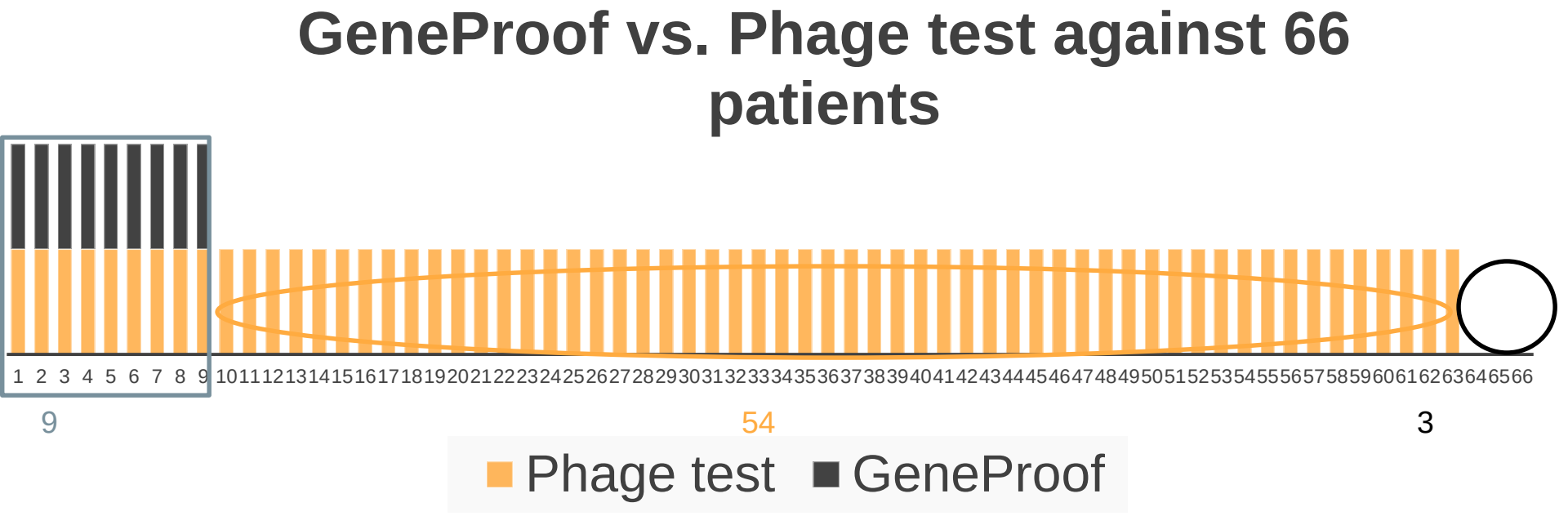
Practical aspects

The test works on whole blood (EDTA coated vials) ; A test performed on whole blood is more sensitive than the one performed on Serum.
We are working to make it available to The test requires 10ml of whole blood (5ml for the test, and 5ml for back-up)

We have selected R.E.D LABS as a partner for it's capability to perform a specific manual DNA extraction followed by qPCR techniques.
R.E.D LABS has a large technical platform in Europe (BRUSSELS) and also an easy to reach facility in the USA (RENO, NV)

Comparison of GeneProof (commercial PCR) and phage tests to determine *Borrelia* presence: Results from 66 patients within the 96-patient cohort

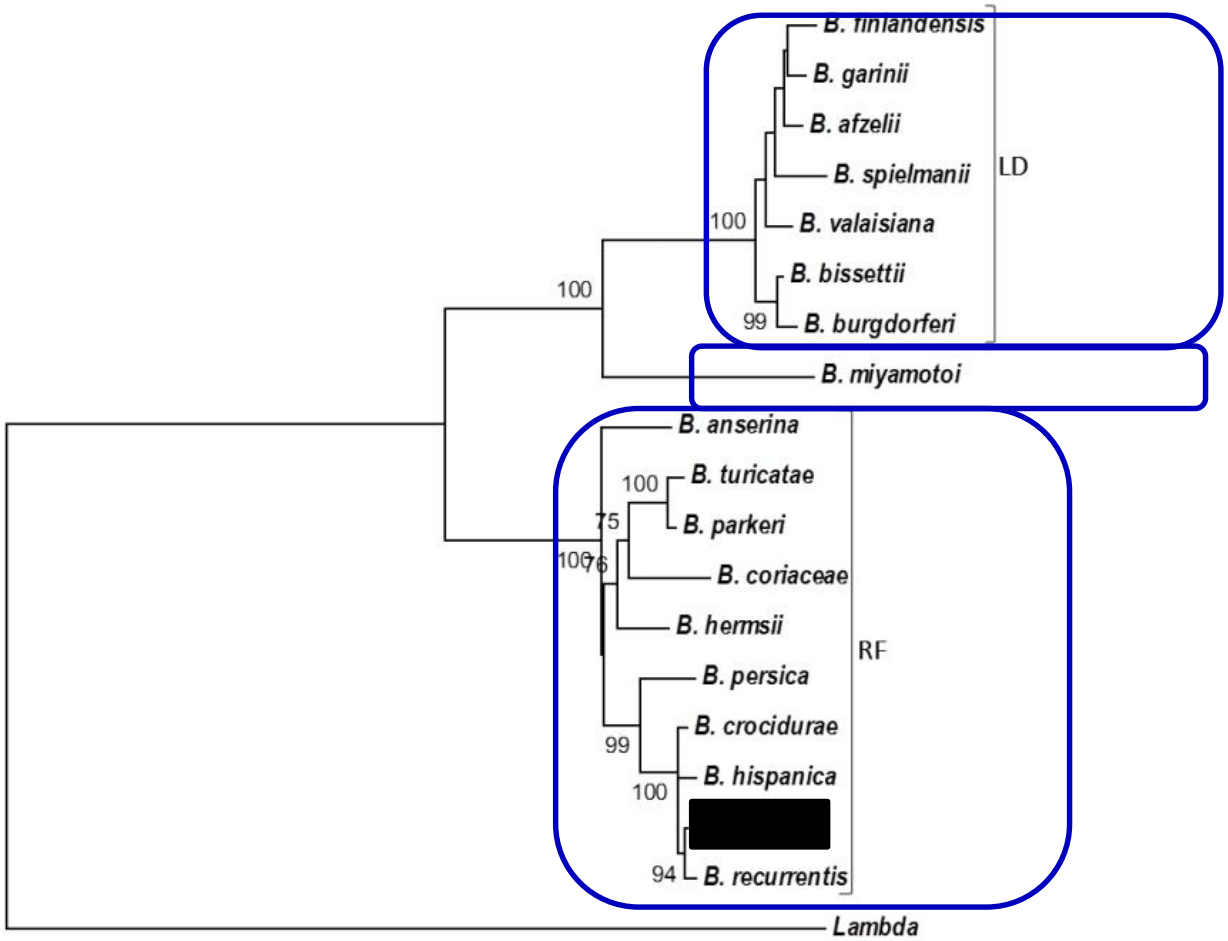
66 patients were random selected from the 96-patient cohort. Nine patients showed both GeneProof and phage positive, while 54 patients showed phage positive and GeneProof negative. 3 patients showed negative for both tests.

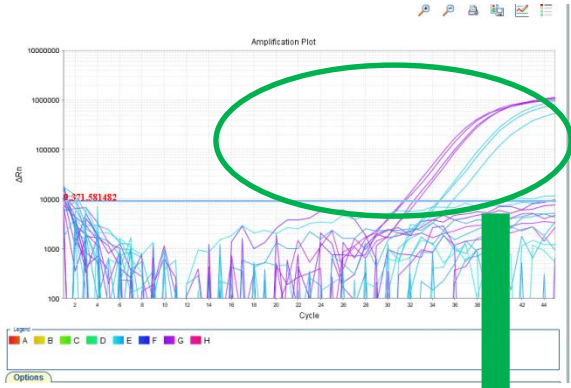


Phelix Phage Test Procedure

Manual DNA Phenol/Chloroform extraction

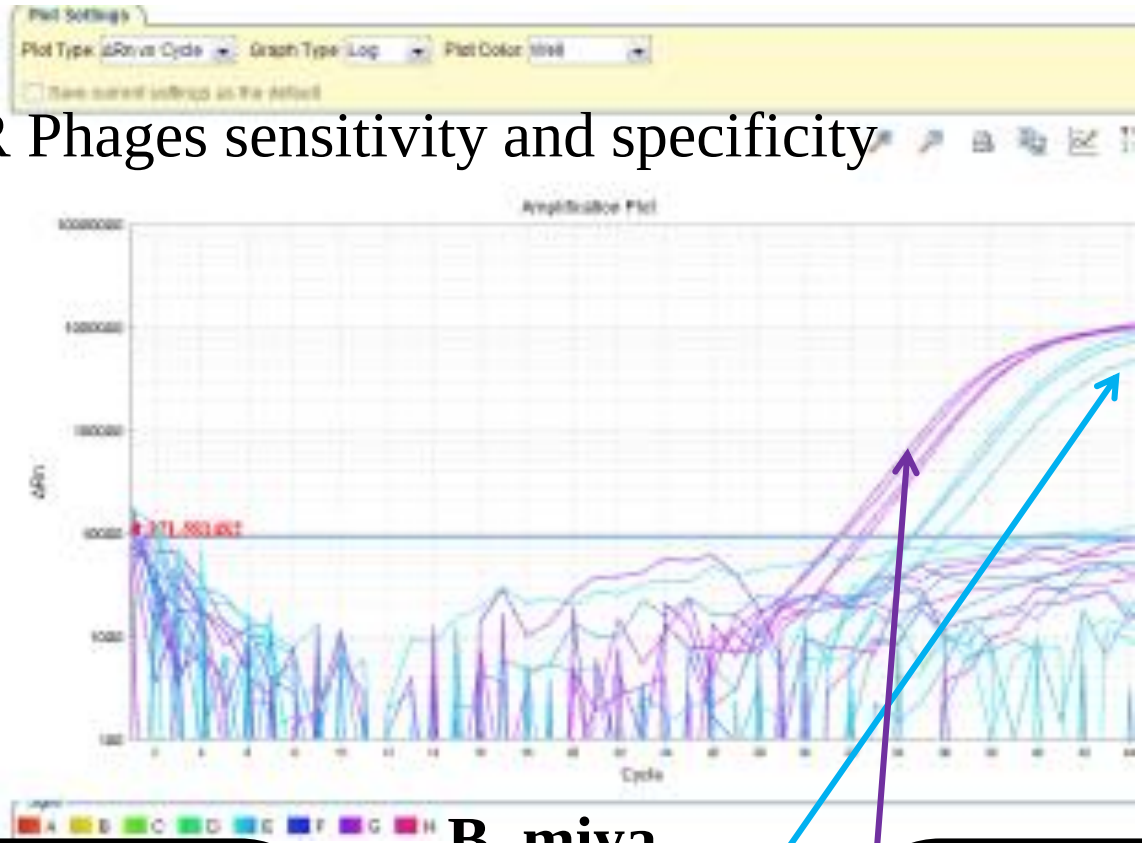
3 different qPCR s





Confirmatory sequencing for positive-like samples

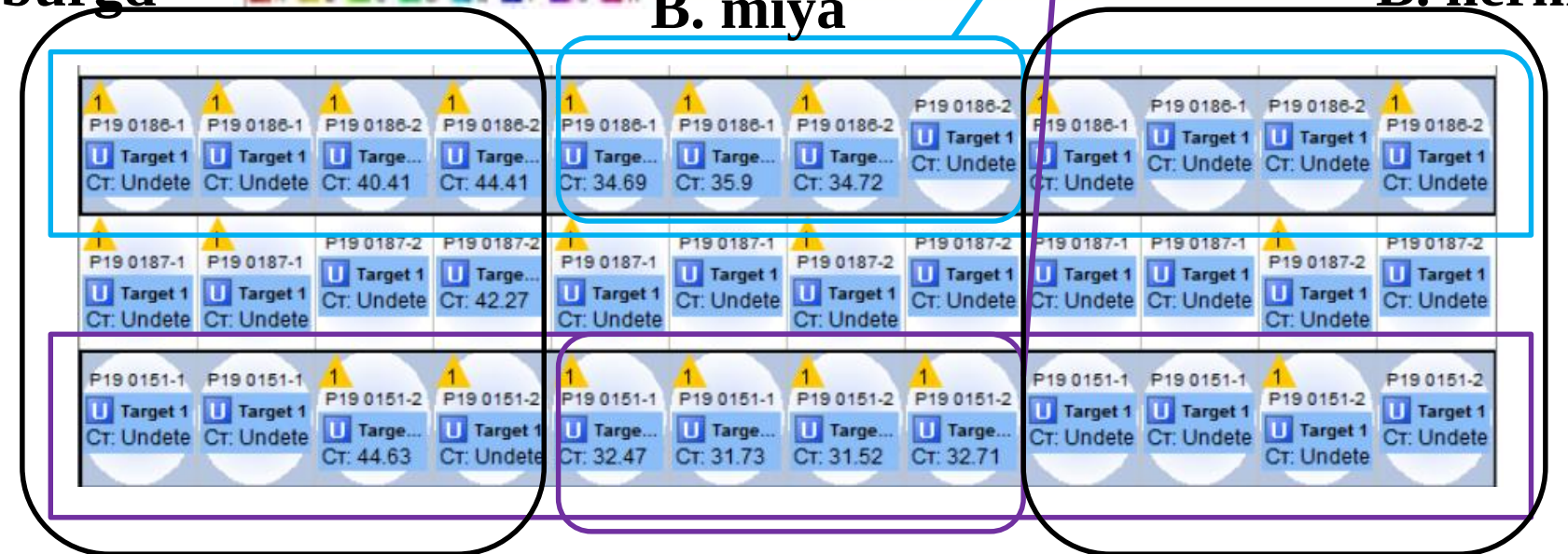
PCR Phages sensitivity and specificity



B. burgd

B. miya

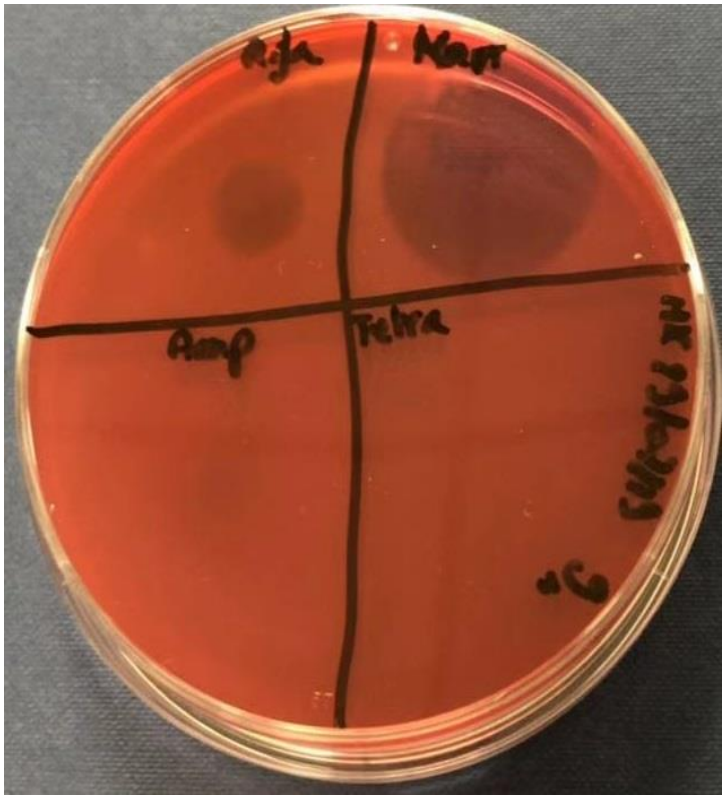
B. hermsii



Future objectives : Develop phage endolysin-based products targeting Borrelia, Bartonella, Mycoplasma, and Rickettsia infections.

- **We have identified Borrelia phage endolysins and** are testing their anti-Borrelia activity in lab using our 'in-house' spot test method. A clear kill zone is revealed with positive results. The same methodology will be applied to analyse Bartonella, Mycoplasma, Rickettsia genomes. Preliminary results showed that several copies of potential endolysins located in Bartonella genomes. Analysis will be conducted to confirm their identity before carrying out protein expression.

Further developments



The antibiotic used for spotting on the Borrelia lawn were 20 µl of tetracycline (100 mg/ml), Ampicillin (100 mg/ml), Kanamycin (50 mg/ml), and Rifampicin (25 mg/ml). Interestingly, it seemed that all the four antibiotics used showed no clear against B Spielmanii.

Kanamycin is much better in killing Borrelia burgdorferi than tetracyclin and Ampicillin. Rifampicin has no visible effect against B. burgdorferi

Kanamycin was twice as effective as Rifampicin in killing Borrelia bisettii, while both Ampicilli no visible effect on B. bisettii.

All four antibiotics can kill B. afzellii

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Bacteriophages are more virulent to bacteria with human cells than they are in bacterial culture ; insights from HT-29 cells
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