Lyme disease: phages for diagnosis and treatment

Louis Teulières³, Jinyu Shan² and Martha Clokie¹

¹PR Head  ²PHD Fellow researcher
Department of Infection, Immunity, and Inflammation, University of Leicester, LE1 7RH, UK

³Infectious and immune diseases consultation
PhelixRD Charity (chronic infections and bacteriophages research group)  louis.teulieres@phelix.org.uk
Disclosure Statement (1):

I do not have any financial arrangements or affiliations with any commercial entities whose products, research, or services may be discussed in these materials.

Please choose one of the above statements to include on your disclosure slide (please make this slide 2 of your presentation).
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.

Inside the spirochaete:

Borrelia have at least 27 plasmids which can be exchanged in whole or part between organisms.
Borrelia (resistance/surviving) forms antibiotics’ lack of efficacy
<table>
<thead>
<tr>
<th>Diagnostics</th>
<th>Qualities and defects</th>
</tr>
</thead>
</table>
| 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 on immune system status and interfering treatments. False positive if time of incubation >24 hours 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?

Bar = 50 nm = 5 \times 10^{-5} \text{ mm}

Magnification of 150,000
Phages are viruses that infect bacteria, and they are everywhere

50 million \((5\times10^7)\) viruses per milliliter of seawater

Estimated number of phages:
The open ocean: \(1.2 \times 10^{29}\)
The soil: \(2.6 \times 10^{29}\)
The ocean sediments: \(3.5 \times 10^{30}\)
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
From Campbell (2003)

Phage life cycle

- Infection
- Attachment
- Bacterium (Escherichia coli)
- Cellular replication
- Induction
- Integration
- Repression
- Lysogenic cycle
- Lysis
- Assembly packaging
- Lytic cycle
- Transcription, translation and replication
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 Prophages.
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***

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

**To engineer phage proteins in killing *Borrelia***

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

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

Holins and endolysins were bioinformatically identified from *Borrelia* genomes.

Borrelia cultures were treated with low concentration of antibiotics to induce phages.
Our Research & Development

We were able to induce Temperate phages from Lyme Borreli strains (table below).

We increased the phage concentration and phage purification.

Phages can be seen under transmission electron microscope (TEM)
Phages residing within *Borrelia* strains are tightly correlated to the identity of their bacterial hosts.
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.

![Phage PCR and bacterial PCR results](image)

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

Doubt means either:
- Negative with Blots
- Negative Border Line

Phage TEST
- 22% NEG
- 78% POS
First results: Phage test versus serological IgM tests

ELISA

Western Blots

Combination WB+ELISA results

Doubt means either:
- Negative with Blots
- Negative Border Line

Phage TEST
## First results: activity detection

Focus on 3 patients with positive results in IgG:

<table>
<thead>
<tr>
<th></th>
<th>ELISA (IgG)</th>
<th>Western Blot (IgG)</th>
<th>Phage test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Patient 2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Patient 3</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

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 negative results in both ELISA and WB IgG and IgM:

- **ELISA IGM + WB IGM**: 59
- **Phage Test**: 48

Phage test detects the presence of active phages in a large number of patients having both negative ELISA and WB (IgG and IgM).
First results: IgM false negative detection

Focus on the patients with negative results in both ELISA and WB IgM only:

Phage test detects the presence of active phages in a large number of patients having both negative ELISA IgM and WB IgM.
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 detecting *Borrelia* in serum collected from 96 patients who were clinically diagnosed as 'Lyme disease' by Dr Louis Teulieres.

<table>
<thead>
<tr>
<th></th>
<th>Phage qPCR results</th>
<th>Serological results</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>88</td>
</tr>
<tr>
<td>Positive</td>
<td>15</td>
<td>73</td>
<td>88</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>96</td>
</tr>
</tbody>
</table>

Phage qPCR: $\frac{88}{96} \times 100\% = 92\%$

Serological assay: $\frac{15}{96} \times 100\% = 16\%$
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.

<table>
<thead>
<tr>
<th>Phage qPCR results</th>
<th>Bacterial qPCR</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
<td>33</td>
<td>55</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>33</td>
<td>63</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Phage qPCR results</th>
<th>Bacterial qPCR</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
<td>31</td>
<td>40</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>31</td>
<td>50</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Phage qPCR results</th>
<th>Bacterial qPCR</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
<td>22</td>
</tr>
</tbody>
</table>

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)

<table>
<thead>
<tr>
<th>Copy number/PCR replicates</th>
<th>Number of PCR positive replicates (% of positive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>10 (100%)</td>
</tr>
<tr>
<td>80</td>
<td>10 (100%)</td>
</tr>
<tr>
<td>60</td>
<td>10 (100%)</td>
</tr>
<tr>
<td>40</td>
<td>10 (100%)</td>
</tr>
<tr>
<td>20</td>
<td>9 (90%)</td>
</tr>
<tr>
<td>10</td>
<td>7 (70 %)</td>
</tr>
<tr>
<td>5</td>
<td>2 (20%)</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>
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 and 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.

**GeneProof vs. Phage test against 66 patients**
Phelix Phage Test Procedure

1. Manual DNA Phenol/Chloroform extraction
2. 3 different qPCR tests
3. Confirmatory sequencing for positive-like samples
PCR Phages sensitivity and specificity
**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 Ampicillin had no visible effect on B. bisettii. All four antibiotics can kill B. afzellii
REFERENCES

Bacteriophage behavioral ecology: How phages alter their bacterial activities. Review article.

Prophage Carriage and Diversity within Clinically Relevant Strains of Clostridium difficile
Jinyu Shan, Krusha V. Patel, Peter T. Hickenbotham, Janet Y. Nale, Katherine R. Hargreaves, Martha R. J. Clokie Environmental Microbiology

Isolation and characterization of temperate bacteriophages of Clostridium difficile.

Phage based diagnosis of bacterial infections
Hari Mohan Saxena Vimlesh Gupta Mini-Review Publish Date: 2016-09-03 Journal of Clinical Trials, Pathology and Case Studies

Development of a Bacteriophage Phage Replication Assay for Diagnosis of Pulmonary Tuberculosis
Ruth McNerney, Bupe S. Kambashi, Juliana Kinkese, Ruth Tembwe, Peter Godfrey-Faussett
ASM DOI: 10.1128/JCM.42.5.2115-2120.2004

Published online 2018 Mar 23. doi: 10.1038/s41598-018-23418-y
PMCID: PMC5865146
PMID: 29572482
Bacteriophages are more virulent to bacteria with human cells than they are in bacterial culture; insights from HT-29 cells
Jinyu Shan, #1 Ananthi Ramachandran, #1 Anisha M. Thanki, #1 Fatima B. I. Vukusic, #1
Dr TEULIERES:
louis.teulieres@phelix.org.uk