Borrelia bacteriophages for diagnosis of Lyme Disease (LD) and Relapsing Fever (RF)

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Facts about Lyme disease (LD) and Relapsing Fever (RF)

- Bacterial infection, caused by *Borrelia* spp.
- Vector-borne disease
- Global distribution
- Problematic in laboratory diagnostics

	Lyme Disease
Causative agent	*Borrelia burgdorferi sensu lato* (*B. burgdorferi sensu stricto*, *B. afzelii*, *B. garinii* etc.)
Vector	hard-bodied ticks (Ixodes spp.)
Diagnosis	Serology and bacterial PCR
Distribution	Worldwide with around 300,000 cases in USA and 65, 000 cases in Europe

	Relapsing Fever
Causative agent	*B. miyamotoi*, *B. anserina*, *B. hermsii*, *B. hispanica*, *B. crocidurae*, *B. turicatae*, *B. parkeri*, and *B. duttonii*
Vector	fox (Pellialus harmanus), hard-bodied ticks (Ixodes spp.) or soft-bodied ticks (Ornithodoros spp.)
Diagnosis	Microscope (blood smear test) and bacterial PCR
Distribution	Worldwide, with estimation of millions of people being infected in Africa

Global distribution of hard-bodied ticks (LD&RF)

(Gerold Stanek, *et al.*, 2012)

Global distribution of soft-bodied ticks (RF)

Lyme disease is one of tick-borne diseases

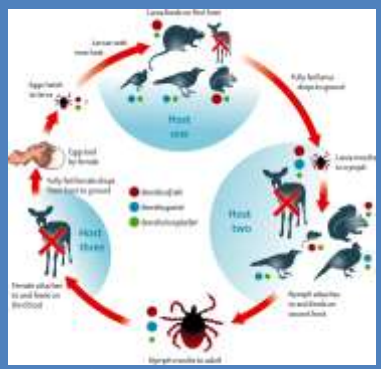
One single tick bite can potentially give you :

Bacterial infection:
- Lyme disease or borreliosis (*Borrelia*)
- Relapsing fever (*Borrelia*)
- Rocky Mountain spotted fever (*Rickettsia*)
- Ehrlichiosis and anaplasmosis (*Rickettsia*-like bacteria)
- Bartonellosis (*Bartonella*)

Viral infection:
- Tick-borne encephalitis
- Powassan virus/deer tick virus

Protozoan (Babesiosis)

Life cycle of typical Lyme *Borrelia* species



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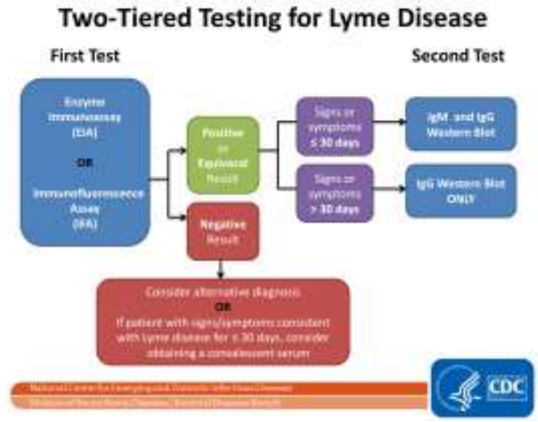


A brief summary of current methods used in detecting *Borrelia*

Diagnostics	Remit
Antibody-based	• Give indirect evidence • Low sensitivity • Can't distinguish active and non-active *Borrelia* presence • Can't distinguish different *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 • Can only detect Lymphocytes that have been in contact with *Borrelia* within 45±15 days, thus limited in application • Distinguish active *Borrelia*? • Distinguish different *Borrelia* sub-types?

A comparison of the current PCR diagnostic methods

Lyme PCR kit	Manufacturer/Inventor	Target sequence	Sample material	Range of detection	Ability to give strain identity	Impact on treatment
GeneProof real time PCR Kit	GeneProof, Czech Republic	16S rRNA	Whole blood	Lyme strains belonging to *B. burgdorferi* sensu lato group	Can't distinguish among Lyme geno-species	In vitro diagnostic
Lyme disease PCR	[illegible] laboratories	No information	Whole blood	*B. burgdorferi* sensu stricto only	Can't detect other Lyme strains	In vitro diagnostic
VetPCR	[illegible] biotechnologies, Inc., China	No information	Animal samples	*B. burgdorferi* sensu stricto only	Can't detect other Lyme strains	Veterinary diagnostic only
B. burgdorferi PCR kit	Norgen Biotek Corporation, Canada	No information	Urine samples	*B. burgdorferi* sensu stricto only	Can't detect other Lyme strains	Research only
[illegible]	[illegible], Germany	No information	Whole blood	Lyme strains belonging to *B. burgdorferi* sensu lato group	Can't distinguish among Lyme geno-species	In vitro diagnostic
[illegible]™PCR	[illegible] Ltd., UK	RecA gene	Human samples including blood	*B. burgdorferi* sensu stricto, *B. afzelii*, *B. garinii*	Can't detect other Borrelia strains, can't distinguish among Lyme geno-species	Research only
PCR Max™ qPCR	PCR Max Ltd., UK	RecA gene	Human samples including blood	*B. burgdorferi* sensu stricto, *B. afzelii*, *B. garinii*	Can't detect other Borrelia strains, can't distinguish among Lyme geno-species	Research only

Bacteriophages (phages) are viruses that infect bacteria


Frederick Twort

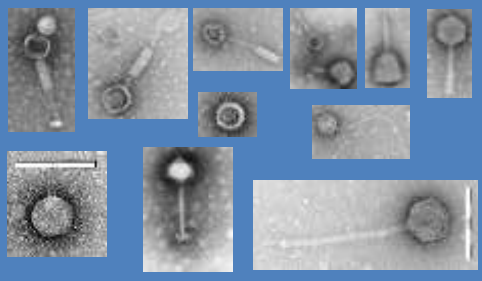

Felix d'Herelle

Phage therapy: therapeutic use of phages to treat bacterial infection

In 1915, the well-known British journal *The Lancet* published an article written by Frederick Twort about "the transmissible bacterial lyses" (Twort, 1915), in which Twort described his observation of "the eaten edges of the colonies of *Staphylococcus*." He managed to filter the appropriate cultures of *Staphylococcus* and spotted the filtrate on the lawn of different *Staphylococcus* strains. Thus, he received a clear zone of lysis again and again. However, Twort could not explain the observed event and provided only its description. This was the very first publication on bacteriophages. d'Herelle read this article, which reminded him of his own observations in Mexico and

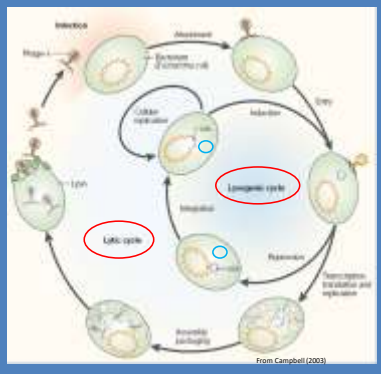
The greatest merit of Felix d'Herelle is that he advanced the idea of using bacteriophages for the treatment of human and animal bacterial diseases. For this idea he deserved the Noble Prize, to which he was nominated eight times, every year since 1925, although he was never awarded one [cited by Hausler (2008) according to Nobel Archives].

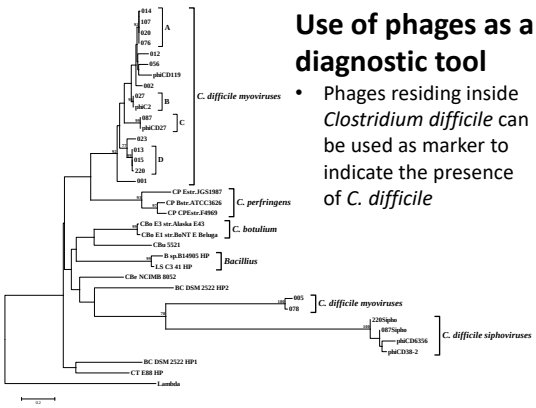
Phage=naked DNA with a protein coat



Phage images taken by transmission electron microscope (TEM) under a magnification of around 100, 000

Life cycle: lytic and lysogenic

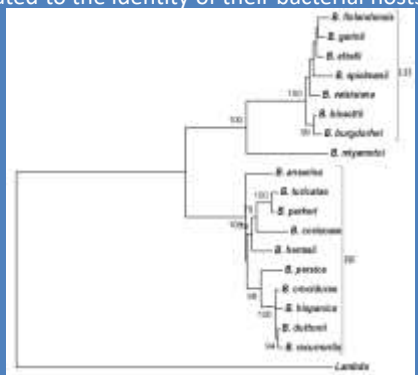


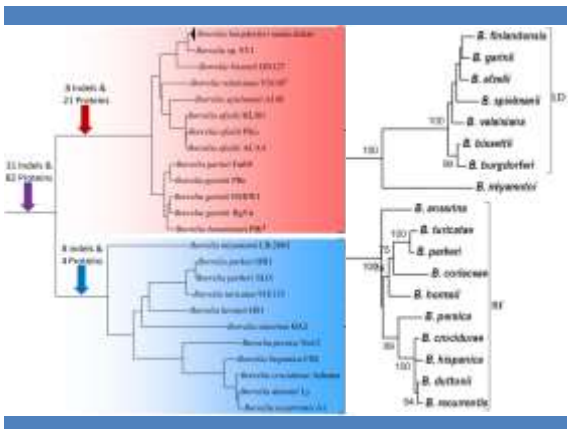


Use of phages as a diagnostic tool

- Phages residing inside *Clostridium difficile* can be used as marker to indicate the presence of *C. difficile*

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





Method development

Phage-based PCR: Journey so far

- 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. 1 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. The size of DNA ladders on both edges of the gel were indicated in bps.

Method development: against five sera

- We applied both phage and bacterial PCRs against five serum samples obtained from Lyme positive patients. Four out of five serum samples generated PCR products with phage PCR (top panel), while only one serum samples produced positive with bacterial PCR (bottom panel).

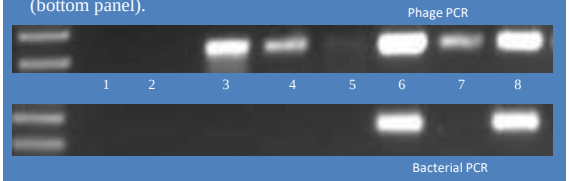
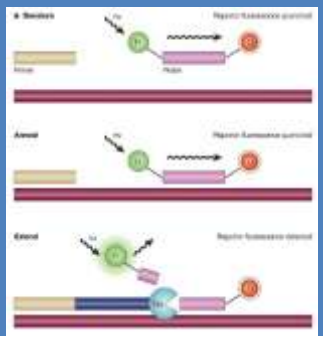


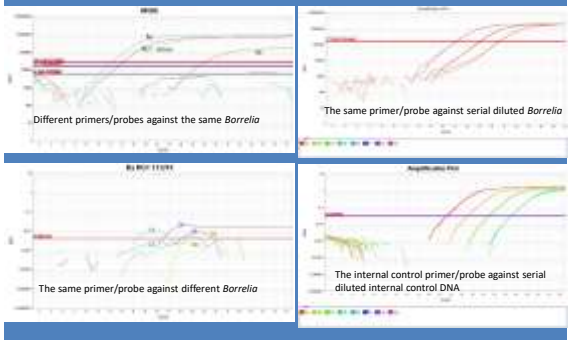
Fig. 3 The phage PCR and bacterial PCRs were carried out against five Lyme positive sera (lanes 3-7) and one serum of healthy volunteer (lane 2). Lane 1 is PCR negative control. Lane 8 is PCR positive control. A single PCR product was generated from Lyme positive serums of 3, 4, 6, and 7 with the expected size and sequence with phage PCR. While only one PCR positives can be seen from bacterial PCR (lane 6). No PCR products from healthy serum and the negative control.

Method development: Taqman-based system

- To further improve the sensitivity and specificity of the phage PCR, a phage-specific primers/probe was designed



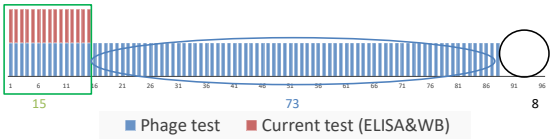
Phage-based PCR amplification curves: positives, negatives, and internal controls



Comparison of the current and phage tests to determine *Borrelia* presence: Results from a cohort of 96 patients

96 patients were examined using the current (ELISA and WB) and phage tests, respectively. 15 patients were positive for both the current and phage tests. 73 patients were positive for phage test, but negative for the current test. Eight patients were negative for both tests.

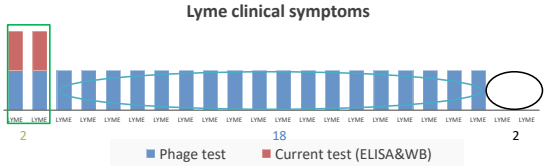
Current test vs. Phage test against 96 patients



Comparison of the current and phage tests to determine *Borrelia* presence: Results from 22 clinically confirmed patients

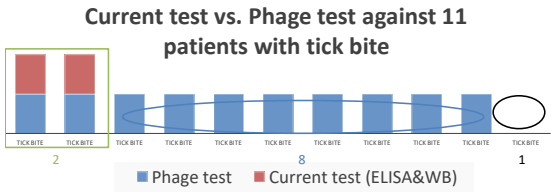
22 patients showed typical 'Lyme clinical symptoms' were examined using the current test and phage test, respectively. Two patients showed both serologic and phage positive. 18 patients showed phage positive but serologic negative. Two patients were negative for both tests.

Current test vs. Phage test against 22 patients with Lyme clinical symptoms



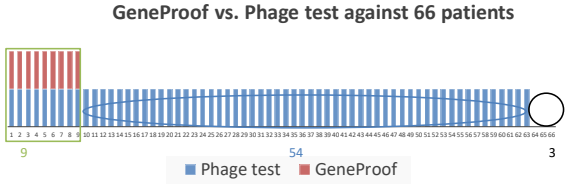
Comparison of the current and phage tests to determine *Borrelia* presence: Results from 11 patients who have been bitten by ticks

11 patients can recall tick bite. Two patients showed both serologic and phage positive. Eight patients were phage positive but serologic negative. One patient was negative for both tests.



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.



How to avoid false positive?

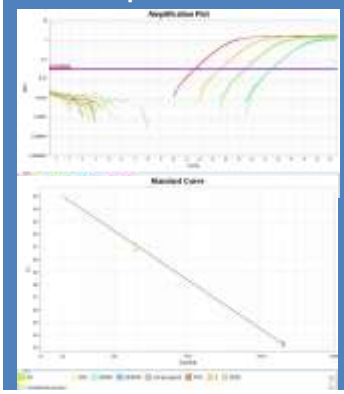
- PCR primers/probes were designed only specific for Lyme *Borrelia.* This was bioinformatically (PCR primer BLAST and *in silico* PCR) and experimentally (targeting various LD&RF *Borrelia* strains) confirmed.
- For every PCR, there were positive and negative controls.
- PCR products generated against serum samples were sequenced and blasted against NCBI database. The top 100 hits were all *Borrelia.*

How to avoid false negative?

False negative:

- For every PCR, there were positive and negative controls.
- An internal control DNA is to be added into each PCR reaction (developing).
- This internal control DNA has been identified, synthesised, primer/probe designed, amplification efficiency determined.

PCR amplification curves: internal controls



The internal control will service as a normaliser to enable a quantitative estimation of 'copy number' of *Borrelia* load.

This will enable phage PCR to measure the relative *Borrelia* load for each case.

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Phage test	• Direct evidence of *Borrelia* presence • High sensitivity and specificity (Pilot study) • Can distinguish Lyme from Relapsing fever *Borrelia* strains • Could distinguish active and non-active *Borrelia* presence (Pilot study) • Could be developed to have the ability to distinguish different *Borrelia* sub-types (ongoing)

Further development

- More controls (healthy and sick) will be needed to figure out the false positive (specificity).
- Random confirmed Lyme patients will be needed to figure out the false negative (sensitivity).
- Primers/probes targeting relapsing fever strain *B. miyamotoi* has been designed, validated in the lab. We are currently testing them against sera.
- A multiplex PCR will be designed to identify LD&RF *Borrelia* strains by a single PCR run.

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Thank you for listening !