Review

Overcoming the Challenges of Lyme Disease Diagnosis: The Role of Phage-based Testing

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Abstract: Tick-borne diseases are a growing concern worldwide, affecting both human and animal populations. Ticks are known to harbor a wide range of pathogens and are considered one of the most important vectors of disease. Lyme disease, caused by *Borrelia burgdorferi* sensu lato, is the most common tick-borne disease in the US and Europe. However, accurate diagnosis of Lyme disease can be challenging due to the complex immune evasion strategies employed by *Borrelia* species and the limitations of existing diagnostic tests. To address this issue, researchers are exploring novel approaches, including the use of bacteriophages as diagnostic tools. Bacteriophages are highly specific and offer advantages over traditional methods for detecting bacteria, including *Borrelia*. In particular, the use of multicopy bacteriophages as molecular markers for *Borrelia* detection is a promising approach that may provide greater sensitivity than targeting single-copy bacterial genes. Nonetheless, the task of identifying trace amounts of bacteriophages in blood samples necessitates attention, and scientists are devising innovative techniques to surmount this hurdle. In summary, employing bacteriophages as a diagnostic tool for tick-borne diseases, such as Lyme disease, by specifically targeting free circulating bacteriophages in blood samples, offers significant potential for enhancing patient outcomes and public health.

Keywords: Tick-borne diseases; Lyme disease; Diagnostic methods; PCR; Bacteriophages (phages); *Borrelia*; Detection limit

1. Introduction

The global burden of tick-borne infections (TBIs) continues to rise, with Lyme disease (LD), caused by the bacteria *Borrelia burgdorferi* sensu lato (s.l.), being the most prevalent TBI in the United States and Europe [1]. Despite the severity of LD and the substantial healthcare expenses linked to delayed diagnosis and treatment, existing diagnostic methods, including the FDA-cleared serological two-tier testing, exhibit limited sensitivity, particularly during the initial phases of the infection when antibodies against *Borrelia* have not yet been generated [2]. This lack of early and effective diagnosis is a major cause of misdiagnosis, leading to long-term patient suffering and contributing to increased healthcare costs.

The prevailing recommendation for laboratory-assisted diagnosis of Lyme disease involves a standard two-tiered (STT) algorithm, which encompasses an enzyme-linked immunoassay (EIA) or immunofluorescence assay (IFA) as the initial test, succeeded by Western blotting (WB) for confirmatory analysis [3]. However, these tests have limited utility during the early stages of infection. Recently, a modified two-tiered (MTT) test offers improved sensitivity and specificity compared to the STT approach, however, it may not detect all early Lyme disease cases, particularly when the immune response is not yet fully developed or if the patient has a weaker immune response to the infection [4, 5].

Given the limitations of indirect antibody-based Lyme diagnostic tests, particularly in detecting early-stage infections and accounting for the complex immune evasion strategies of the bacteria, there

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is a pressing need for direct testing methods. Direct tests, which target the Lyme-causing bacteria itself, can provide more accurate and timely diagnosis, facilitating prompt treatment and improved patient outcomes. PCR-based assays have emerged as alternative tests in research settings, directly detecting infectious agents. For example, using PCR targeting the *oppA1* gene of *B. burgdorferi* sensu lato, researchers at the Mayo Clinic identified a novel *Borrelia* species causing LD in the USA [6] . This new pathogenic genospecies (candidatus *Borrelia mayonii*) results in unusually high spirochaetaemia, emphasizing the importance of *oppA1* PCR for accurate diagnosis and raising awareness of its distinct clinical features [6]. These tests emphasize the importance of developing innovative diagnostic approaches to improve patient outcomes. One promising method under investigation involves using *Borrelia*-specific bacteriophages , also known as phages, for LD detection, potentially enhancing accuracy and efficiency.

Phages, viruses that specifically infect bacteria, have gained considerable attention for their potential use as markers in detecting bacterial infections. This is particularly true for prophages, which reside within the bacteria themselves. The interest in phage-based diagnostic tools stems from their tight correlation with their bacterial hosts and their potential to be present in higher concentrations compared to bacterial cells [7-9]. As a result, researchers are focusing on developing novel phage-based diagnostic tools for various bacterial infections. One example of using phages as markers to detect bacterial infections is the use of phage-based PCR to detect *Mycobacterium tuberculosis*, which enables faster results and identification of drug-resistant strainsfilamentous phages [10, 11].

The primary objective of this review is to explore the potential of phage-based diagnostics in the laboratory diagnosis of LD, exemplified by a case study employing *Borrelia*-specific phages as a diagnostic tool. By capitalizing on the free-circulating nature of phages and the robustness of real-time PCR, this approach offers several advantages over traditional diagnostic methods. First, due to the higher abundance of phages relative to bacterial cells, this method demonstrates greater sensitivity compared to conventional bacteria-targeting PCR tests. Second, the phage-based test can discriminate between various *Borrelia* subtypes, such as *B. burgdorferi* sensu lato and *B. miyamotoi*, as phages function as indicators of bacterial identity. Lastly, this test exhibits potential for detecting LD across a wide range of infection stages, from early to late, thereby improving diagnostic accuracy and providing valuable information for effective patient management.

2. The Growing Threat of Tick-Borne Diseases (TBDs): Understanding the Complexities, Impact, and Approaches to Diagnosis and Treatment

Ticks, belonging to the Acari subclass, are obligate hematophagous arthropods that primarily feed on the blood of vertebrate animals. These parasitic creatures occasionally bite humans as well, resulting in potential transmission of various pathogens and diseases [12-14]. TBDs present a significant and escalating threat to human and animal populations worldwide, with an estimated global burden affecting over half a million individuals [15-17] . The health impacts of TBDs extend beyond the direct consequences of infection, as they also contribute to substantial healthcare costs, productivity loss, and long-term disability in some cases [1]. The increasing prevalence of TBDs has amplified the need for improved diagnostic methods and targeted interventions to reduce the health and economic burdens associated with these diseases. As the incidence of TBDs continues to rise, it is crucial to address these challenges through innovative research and the development of effective strategies for prevention, diagnosis, and treatment.

Climate change, including global warming, has been linked to the expansion of tick populations worldwide, contributing to the increased prevalence of TBDs [18]. The tick life cycle typically spans two years, depending on host availability, and involves three stages: larva, nymph, and adult [19]. Ticks acquire pathogens when feeding on infected hosts and subsequently transmit infection during future bites. Optimal conditions for ticks and reservoir hosts increase the number of infected ticks, elevating the risk of TBDs, including LD [20].

LD is caused by a group of bacteria called *Borrelia burgdorferi* s.l. complex, which encompasses a group of over 20 distinct genospecies of bacteria. Among these, three dominant genospecies—*B. burgdorferi* sensu stricto (s.s.), *B. garinii*, and *B. afzelii*—are primarily responsible for causing LD. *B.*

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burgdorferi s.s. is predominantly found in the United States, whereas *B. garinii* and *B. afzelii* are more commonly present in Europe and Asia [21-23]. It is important to note that the term ′*B. burgdorferi* s.l.' used throughout this manuscript refers to a collection of *Borrelia* species exhibiting genomic variations, rather than a single, uniform species [21]. Lyme disease is the most prevalent vector-borne disease in the US, with an estimated 476,000 annual cases, and affects over 200,000 individuals per year in Western Europe[15, 16]. Understanding the diversity and distribution of *Borrelia* species causing LD is crucial for effective diagnosis and treatment. Equally important is the emerging recognition of another *Borrelia* species, *B. miyamotoi*, which has been garnering increased attention in recent years

B. miyamotoi, first identified in 1995 in ticks from Japan, has since been found worldwide [24]. The notable rise in documented cases of both tick carriage and human infections involving *B. miyamotoi* demonstrates a significant trend [25]. This bacterium is classified as a spirochete causing tick-borne Relapsing Fever (RF), a disease recognized since the time of Hippocrates in ancient Greece [26]. Members of the RF clade, mainly transmitted by soft-bodied Argasidae ticks, have been less extensively studied than those of the LD clade.

Accurate differentiation of LD stages (early localized, early disseminated, and late disseminated) is vital for appropriate treatment stratification [21]. Early-stage treatments usually involve a shorter course of antibiotics, while late-stage or chronic Lyme disease may necessitate extended treatment and additional supportive therapies [27, 28]. However, a subset of patients may continue to experience symptoms after receiving appropriate treatment, referred to as chronic Lyme disease or Post-Treatment Lyme Disease Syndrome (PTLDS) [29-31]. Dr. Horowitz recommends using the term Lyme-MSIDS (Multiple Systemic Infectious Disease Syndrome) to acknowledge the complexity of chronic Lyme disease and other co-infections transmitted by ticks, reflecting the multifaceted nature of the disease involving various pathogens, immune dysfunction, and other contributing factors [32]. Adopting this broader perspective could help guide more effective diagnosis and treatment strategies for patients suffering from chronic Lyme disease or other tick-borne co-infections. Recognizing and addressing these stages can significantly improve patient outcomes and prevent long-term complications.

3. Advancements in Lyme Disease Diagnostics: Overcoming Limitations of Serological Testing with Direct Detection Methods

LD diagnostics primarily involve a combination of direct detection methods, which identify the infectious agent, and indirect detection methods that detect a host response to the infection. Serology, the most common indirect method, employs a two-tiered testing algorithm consisting of an enzyme-linked immunosorbent assay (ELISA) screening test, followed by a highly specific immunoblot test [27]. Both tests are antibody-based, offering the advantage of detecting a host's immune response to the infection. While ELISA is relatively insensitive, it can still detect a significant proportion of infected patients, providing valuable information for clinical decision-making. The immunoblot test serves as a complementary tool, offering high specificity to confirm positive or equivocal ELISA results. Together, these tests can help diagnose LD in situations where sufficient antibodies have been generated. However, during the first four-to-six weeks of infection, patients may not have developed a sufficient antibody response, limiting the utility of these tests [33]. In such cases, there is a need for direct and sensitive diagnostic methods that can detect the presence of the infectious agent itself, ultimately improving patient care and management [34].

The advancements in scientific methodologies and techniques have paved the way for the exploration of direct testing options for LD. These direct tests, which target bacterial proteins or nucleic acids, demonstrate the potential to accurately identify the presence of the causative *Borrelia* species without relying on the host's immune response. As a result, direct testing methods could significantly enhance the diagnostic process by overcoming the limitations of serological tests, particularly during the early stages of infection when antibody responses may be insufficient. The development and implementation of direct testing methods for LD, based on rigorous scientific evidence, may ultimately lead to more effective diagnosis, treatment, and management of the disease.

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Direct testing methods for LD, such as culture, antigen-based methods, and nucleic acid-based methods, offer varying degrees of sensitivity and practicality, with ongoing advancements in each field striving to improve diagnostic capabilities. Culturing *B. burgdorferi* is time-consuming and impractical for routine use, as it requires special media and takes up to 12 weeks to grow, with relatively low sensitivity [35]. Antigen-based methods, such as antigen-capture assays, directly detect bacterial antigens shed or secreted into body fluids. However, developing a sensitive and specific antigen-capture assay for Lyme disease has been challenging due to poor specificity, low sensitivity, and the difficulty of choosing an appropriate capture antigen target [34, 36]. Advances in proteomics, specimen processing, mass spectrometry, and emerging antigen concentration and enrichment methods can potentially overcome these limitations [34, 37]. Nucleic acid-based methods, such as PCR assays, directly detect pathogen DNA. While standard PCR methods have limited sensitivity for LD, due to low numbers of *B. burgdorferi* in tissue and body fluids, various strategies can improve sensitivity without compromising specificity [33, 34, 38, 39]. These include starting with larger specimen volumes, using target enrichment methods, and employing unconventional signal detection methods, including targeting multicopy *Borrelia* phage genes [40-42]. This direct detection is particularly essential for recognizing various Borrelia strains that might not be captured through standard serology testing. PCR strategies have performed well in blood tests for other infections, and enhancement and enrichment methods can make *B. burgdorferi* detection in blood more feasible [43, 44]. Ultimately, the development of more sensitive PCR assays for Lyme disease may benefit from combining these various strategies to maximize detection capabilities.

The diagnosis of LD faces significant obstacles due to the complex immune evasion strategies employed by *Borrelia* species and the inherent limitations of existing tests. There is a pressing need for the development of a reliable diagnostic test for LD, as delayed diagnosis contributes to increased healthcare costs and adverse patient outcomes. To address this issue, it is crucial to explore novel, highly sensitive testing methods. One such promising approach currently under investigation involves the utilization of *Borrelia*-specific phages as a diagnostic tool, which holds the potential to improve the accuracy and efficiency of LD detection [8, 9, 42].

4. Overcoming Challenges in Detecting Low-Level PCR Template in Human Samples: Strategies and Insights from Forensic Science

PCR-based methods are widely used for the detection of infectious agents in clinical and research settings. However, the sensitivity of PCR assays can be limited, particularly when the target DNA is present at low concentrations, such as in human blood and serum samples. The challenges of detecting low levels of PCR template in these samples are multifactorial and have been the focus of extensive research in recent years [40, 42, 45, 46]. One major factor that contributes to the difficulty in detecting low levels of PCR template is the presence of inhibitors in human samples [47]. These inhibitors can affect the efficiency of PCR amplification and lead to false-negative results. Inhibitors can be introduced during sample collection, processing, and storage, or they can be endogenous to the sample itself. Common inhibitors of PCR include heme, heparin, and immunoglobulins [47-49].

Several methods have been developed to overcome the problem of PCR inhibition in human samples. One approach is to use specialized PCR reagents that are designed to neutralize or remove inhibitors [48, 49]. For example, some commercial kits contain reagents that remove heme from blood samples or remove PCR inhibitors from serum samples. However, these methods can be expensive, time-consuming, and may not remove all inhibitors.

Another approach to detecting low levels of PCR template is to use more sensitive detection methods, such as digital PCR (dPCR). dPCR allows the absolute quantification of nucleic acid targets without the need for standard curves [50]. dPCR has been applied to detecting *B. burgdorferi* with the aim of improving diagnostic sensitivity and specificity. One study sought to establish a dPCR assay for detecting *Borrelia* in cerebrospinal fluid (CSF) samples, focusing on optimizing pre-PCR procedures [51]. Although the optimized *Borrelia*-specific dPCR method demonstrated a high specificity, the diagnostic sensitivity was found to be low, leading the researchers to conclude that it may not be suitable as a routine diagnostic method for LD. In another study, researchers developed a dPCR assay

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that achieved a two-fold higher sensitivity compared to existing diagnostic methods by using a larger sample volume, applying pre-analytical processing to blood samples, and implementing a pre-amplification step to enrich *B. burgdorferi*-specific gene targets [52]. This approach showed promise for detecting LD at the onset of symptoms, potentially enabling more timely and effective treatment, limiting antibiotic overuse, and reducing associated morbidities. Although dPCR can be a sensitive method for detecting low levels of PCR template, the cost of reagents and equipment for dPCR can be higher than traditional PCR assays, making it less accessible for some laboratories.

Alternatively, another approach that can be used is next-generation sequencing (NGS), which allows for the analysis of millions of DNA fragments simultaneously, providing a highly sensitive and specific method for detecting low-level targets [53]. Unlike PCR, which amplifies specific DNA regions, NGS can sequence all DNA fragments present in a sample, allowing for the detection of even highly divergent target sequences. Additionally, NGS can identify multiple targets in a single sample, making it a powerful tool for detecting complex infections or multiple pathogens simultaneously [53, 54].

Several studies have demonstrated the potential of NGS in detecting low-level targets in human samples. For example, NGS has been used to detect rare mutations in circulating tumor DNA, allowing for the early detection of cancer recurrence [55]. Another study demonstrates that NGS has the potential to identify *B. burgdorferi*, from tick samples [56]. This molecular approach could improve early diagnosis and treatment, overcoming the limitations of current diagnostic tests. NGS has the potential to improve LD detection by sequencing millions of DNA strands, including *B. burgdorferi*, from blood samples. Further studies will be needed to compare NGS with standard serologic testing, exploring its sensitivity and the impact of antibiotics on its performance in detecting LD across various stages [57, 58].

However, there are also some limitations to using NGS as an alternative to PCR for detecting extremely low targets. NGS can be expensive, time-consuming, and requires specialized equipment and expertise. Additionally, NGS generates vast amounts of data, which can be difficult to analyze and interpret, leading to increased costs and potential errors.

Forensic science has also provided insights into the challenges of detecting low levels of DNA in human samples [59]. In forensic investigations, trace amounts of DNA from human biological samples, such as blood and semen, are often the only source of DNA available for analysis. Therefore forensic scientists have developed methods to detect DNA at extremely low levels, including more sensitive DNA extraction methods, the incorporation of amplification controls to detect PCR inhibitors, and the use of mini-STR and multiplex PCR for STR analysis [59, 60]. These methods have allowed for the detection of DNA at extremely low levels in forensic samples and have important implications for criminal investigations and justice.

One example of a forensic DNA analysis method that has been developed to detect DNA at extremely low levels is the mini-STR analysis. Mini-STR analysis is a modification of standard short tandem repeat (STR) analysis, which is widely used in forensic DNA typing. Mini-STR analysis targets smaller STR loci than standard STR analysis, which allows for the amplification of shorter DNA fragments and may improve the detection of degraded or low-level DNA samples [61]. Another example is the use of multiplex PCR for STR analysis, which allows for the amplification of multiple STR loci simultaneously. This technique has been shown to be more sensitive than singleplex PCR, allowing for the detection of DNA samples with lower DNA concentrations [62]. In addition to these methods, forensic scientists have also developed more sensitive DNA extraction methods, such as the use of magnetic bead-based DNA extraction kits, and the incorporation of amplification controls to detect PCR inhibitors, such as the TaqMan PCR inhibitor assay [63, 64].

Overall, the development of these methods has allowed for the detection of DNA at extremely low levels in forensic samples, which has important implications for criminal investigations and justice. Similarly, the application of sensitive PCR techniques in other fields, such as LD diagnostics, may also lead to improved detection of low-level targets and ultimately better patient care.

In conclusion, the detection of low levels of PCR template in human samples, such as *Borrelia* DNA, remains a challenge in clinical and research settings. However, various techniques,

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methodologies and the lessons learned from forensic science can be applied to the detection of LD. Some key methods and strategies to consider could include:

1. Specialized PCR reagents: Using PCR reagents designed to neutralize or remove inhibitors, such as heme, heparin, and immunoglobulins, can help to overcome PCR inhibition in human samples.
2. dPCR: It allows for absolute quantification of nucleic acid targets without the need for standard curves, making it a more sensitive detection method than traditional PCR assays. By optimizing pre-PCR procedures and applying pre-amplification steps, dPCR can achieve higher diagnostic sensitivity and specificity for detecting *B. burgdorferi*.
3. NGS: It is a highly sensitive and specific method for detecting low-level targets. It enables simultaneous analysis of millions of DNA fragments and can identify multiple targets in a single sample.
4. Forensic DNA analysis techniques: Incorporating methods developed in forensic science, such as, multiplex PCR and sensitive DNA extraction methods like magnetic bead-based DNA extraction kits, can improve the detection of low-level DNA samples. Additionally, using amplification controls to detect PCR inhibitors can enhance the diagnostic process.

To develop a sensitive antigen-based Lyme detection method, a combination of the aforementioned approaches could be employed. This would potentially result in a more accurate and effective diagnostic tool for LD, leading to better patient care and more timely treatment.

5. Harnessing Free Circulating Phages as Diagnostic Markers for Bacterial Infections: Advancements and Future Perspectives

Phages, viruses that infect bacteria, are not only useful as therapeutic agents but also as molecular markers for bacterial identification [9, 42, 65]. These phages possess highly specific host preferences, allowing for the development of phage-based molecular markers for bacterial identification. Phage-based molecular markers offer several advantages over traditional methods, including their speed, specificity, and sensitivity [9, 42, 66]. One growing area of interest is the role of free circulating phages in body fluids and their potential as diagnostic tools.

Phage-based bacterial detection methods, such as plaque assays, phage amplification assays, phage-based biosensors, and phage-display technology, have been successfully applied to identify various bacterial pathogens [67-69]. These methods offer rapid detection with high specificity and sensitivity in diverse sample types. For example, phage-based PCR has been used to detect *Mycobacterium tuberculosis*, responsible for causing TB. This approach combines phage specificity with PCR sensitivity, offering faster results and the ability to identify drug-resistant strains, making it a promising addition to TB diagnostics, particularly in resource-limited settings [10, 70].

One potential application of phages is their detection in free circulating form in body fluids. Recent studies have shown the presence of free circulating phages in various body fluids, including blood, urine, and saliva, with potential biological functions [71]. These phages may play a role in modulating the human microbiome by selectively targeting certain bacteria or by transferring genetic material to host bacteria through horizontal gene transfer [72]. Additionally, free circulating phages could serve as biomarkers for bacterial infections, providing a non-invasive and sensitive diagnostic tool. In this context, the detection of free circulating phages could potentially be exploited to develop molecular tests for various bacterial infections. One study found that free circulating phages in blood

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samples could distinguish between patients with sepsis and healthy controls, indicating their potential as diagnostic markers for sepsis [73].

To target these free circulating phages and develop molecular tests, scientists need to identify specific phage markers that correlate with the presence of a particular bacterial pathogen. High-throughput sequencing technologies and bioinformatics tools could help identify these markers, which could then be targeted using (PCR) or other molecular techniques [74, 75]. By employing phage-specific primers and probes, these tests could offer high specificity and sensitivity in detecting bacterial infections. Moreover, the development of rapid and user-friendly techniques, such as isothermal amplification or point-of-care devices, could enable the detection of free circulating phages in clinical settings, even in resource-limited environments [76, 77]. This would allow healthcare professionals to diagnose bacterial infections more quickly and accurately, leading to timely and appropriate treatments. Furthermore, monitoring the dynamics of free circulating phages in response to antibiotic therapy could provide valuable information about treatment efficacy and the development of antibiotic resistance [78]. This knowledge could aid in the optimization of treatment regimens and the implementation of personalized medicine approaches.

As more sequencing data becomes available, researchers can better characterize the 'phagenome' – the complete collection of phages present in the human body [79]. This expanded knowledge of the human phagenome will increase the feasibility of PCR-based approaches to probe free circulating phages as diagnostic markers. The identification of specific phage sequences related to different bacterial infections can significantly improve the specificity and sensitivity of phage-based molecular tests.

In conclusion, free circulating phages in body fluids offer immense potential as diagnostic markers for bacterial infections due to their specificity, sensitivity, and potential biological functions. Phage-based molecular markers offer advantages over traditional methods, and phage-based bacterial detection methods have been successfully applied to identify various bacterial pathogens. As researchers continue to refine phage-based detection techniques and tackle associated challenges, these methods hold promise in revolutionizing the detection and monitoring of bacterial infections, ultimately benefiting patients and public health worldwide.

6. Phage-based Detection and Identification of *Borrelia* Species: Overcoming Challenges and Advancing Diagnostic Capabilities

The specificity of bacteriophages in targeting a single bacterial species forms the foundation for using phages as proxies for bacterial presence. A strong correlation between phages and their corresponding bacterial hosts has been well-established in scientific literature, as evidenced by numerous publications [8, 9, 42, 65, 80, 81]. Progress in developing phage-based molecular markers for bacterial identification has been significantly influenced by our growing understanding of *Borrelia* phages, which are associated with LD. This increased knowledge enables scientists to better detect and identify the presence of Borrelia species using these phages as markers.

Unlike traditional molecular markers, such as 16S ribosomal DNA, phages do not possess an equivalent gene, making gene selection for amplification and primer design in phage PCR challenging. However, common phage genes used as molecular markers include those encoding major capsid protein, portal protein, DNA polymerase, and terminase [9, 42, 82-85]. As more sequencing data becomes available, the feasibility of PCR-based approaches to probe the 'phagenome' will increase [72].

Unique phage sequences present in *Borrelia* species serve as effective indicators for bacterial identification, providing a valuable proxy for detecting these bacteria. With multiple phage genes present per *Borrelia* cell, the detectable signal is higher for phages than for bacteria [8, 42]. Experimental evidence has shown that *Borrelia* phages can be released outside the *Borrelia* cells [42, 86-88]. By leveraging the multicopy nature and mobility of *Borrelia* phages, scientists can bypass the elusive and tissue-embedded properties often associated with human *Borrelia* infections, thus improving detection and identification [89, 90].

Employing phage markers for bacterial detection presents a significant advantage, particularly for tissue-bound bacteria like some *Borrelia* species, which only transiently circulate in the blood.

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Since phages can be released from their host bacteria, they can freely move and potentially access areas where bacteria are present, thereby improving detection capabilities. To test this hypothesis, we compared a phage-based test with a chromosomal-based method for detecting *Borrelia* with *Borrelia*-spiked human blood. Our study demonstrated that the phage-based diagnostic test had a higher sensitivity compared to the 16S ribosomal DNA-based method in detecting *Borrelia*, with a detection limit approximately 10-fold lower [8, 42].

To validate the use of a phage-based method for detecting *Borrelia* in blood, it is crucial to directly isolate *Borrelia* phages from blood samples. This approach provides direct evidence of the existence of phages that are specific to *Borrelia* in the bloodstream. Although *Borrelia* phages can be present in either a free circulating form or as plasmid/temperate phages within *Borrelia* cells, isolating these phages from blood samples will enable the identification and characterization of specific phages that infect *Borrelia*. This information is essential for selecting the optimal phages for use in phage-based detection assays. Additionally, characterizing *Borrelia* phages will provide a better understanding of the interaction between phages and *Borrelia*, which may have implications for the development of phage therapy for *Borrelia* infections. Furthermore, if *Borrelia* phages can be isolated from blood samples, it would demonstrate the presence of free circulating phages in blood, providing evidence that phages might have potential biological functions in the human body [91]. Such information could inform the development of novel diagnostic tools and therapeutic interventions based on phage biology.

However, isolating phages from blood samples, including *Borrelia* phages, can be challenging due to factors such as low phage concentrations in the blood and potential presence of inhibitors that could interfere with phage isolation and detection. Blood components like heparin, EDTA, or antibodies may affect the ability to culture or amplify phages, making their isolation from blood samples more difficult. Culture-based methods like plaque assays are commonly used to isolate phages, but the intrinsic difficulty in culturing *Borrelia* makes this approach challenging for identifying free circulating Borrelia phages in blood samples [35, 52, 92, 93]. Consequently, plaque assay-independent methods, such as PCR-based approaches, are being developed to overcome this challenge.

While free phages have been discovered in some clinical samples, including blood, isolating *Borrelia* phages from blood samples is still an ongoing effort [72, 91]. Despite these challenges, the potential advantages of using phages as a proxy for bacteria make it a promising area for continued research and development. As scientists continue to explore phage biology and its applications in detecting and identifying bacteria, phage-based methods may offer more sensitive and effective diagnostic tools for bacterial infections, such as those caused by *Borrelia* species.

7. Overcoming Detection Limit Challenges in ROC Curve Analysis for Phage-based qPCR Diagnosis of Borrelia Infections

To develop a reliable phage-based PCR method for detecting Borrelia in blood, it is essential to evaluate the diagnostic accuracy of the test. The receiver operating characteristic (ROC) curve is widely used to assess the sensitivity and specificity of a binary classifier system at different threshold settings [94]. The area under the ROC curve (AUC) serves as a measure of the overall diagnostic accuracy, with an AUC value of 1.0 representing a perfect test and a value of 0.5 indicating random guessing [95].

In clinical practice, many diagnostic tests have detection limits below which values cannot be accurately quantified. This can be attributed to factors such as test sensitivity, the availability of sample material, or the presence of inhibitors within the sample. When dealing with low levels of bacterial or viral DNA, values below the detection limit can pose challenges in constructing ROC curves, potentially biasing the results or impacting the test's accuracy. One example of this issue occurs when detecting *Borrelia* using phage-based qPCR (Ter-qPCR) [42]. It is well-established that the number of *Borrelia* cells circulating in the blood is extremely low, often at the lower end of the qPCR detection limit [44, 96]. A low number of PCR templates can lead to qPCR variability due to stochastic effects, such as a single successful qPCR among multiple technical repeats [44, 97]. Shan et al. demonstrated that one copy of PCR template led to two positives out of 10 replicates when using Ter-qPCR, while

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20 and 40 copies generated nine and ten positives out of ten replicates, respectively. In their study, Shan et al. observed that among six technical repeats obtained from one LD patient's blood sample, the Ter-qPCR showed varying copy numbers of the phage terminase gene. When this data was used to construct a ROC curve, the analysis failed to provide a valid estimation of the test's true diagnostic properties, which aligns with previous reports (Perkins et al., 2006; Bantis et al., 2017).

Several approaches have been proposed to address the challenges posed by values below the detection limit in ROC curve analysis. These include utilizing imputation methods to estimate values below the detection limit [98] and developing nonparametric methods for ROC curve analysis capable of handling censored data [95]. Alternatively, focusing on improving sample preparation steps to increase copy number values can help overcome the limit of detection (LoD) [99]. Enhancements in sample preparation will yield more reliable data, enabling the construction of a valid ROC curve and accurate estimation of the test's true diagnostic performance [99] .

In conclusion, ROC curve analysis is a valuable tool for evaluating the performance of diagnostic tests; however, its accuracy can be impacted by values below the detection limit. As diagnostic tests continue to improve in sensitivity and accuracy, it is crucial to consider the effects of values below the detection limit on ROC curve analysis and develop new approaches to address these challenges.

8. Discussion

TBDs are a growing concern globally, with over half a million estimated cases worldwide. Ticks transmit a wide range of pathogenic microorganisms, and LD is the most prominent TBD, affecting hundreds of thousands of people annually in the US and Western Europe. The diagnosis of LD can be challenging, and there is a pressing need for more reliable and sensitive diagnostic tests. One promising approach is the use of *Borrelia*-specific phages as a diagnostic tool. Phages can also serve as molecular markers for bacterial identification, and their specificity makes them a promising tool for detecting bacterial infections. The use of phages as a proxy for detecting *Borrelia* infections is based on the close correlation between phages and their corresponding bacterial hosts, with *Borrelia* carrying specific phage sequences that can be utilized as a proxy to identify the bacteria. However, detecting *Borrelia* phages in blood samples can be challenging due to several factors, including low phage concentration and the potential presence of inhibitors. Additionally, values below the detection limit can impact the accuracy of diagnostic tests, making it important to consider the impact of such values on test performance evaluation. Overall, continued research and development of novel approaches are necessary to overcome these challenges and improve the diagnosis and management of tick-borne diseases.

9. Conclusions

In conclusion, TBDs pose a significant and growing concern for human and animal populations worldwide, with LD being the most prevalent vector-borne disease in the US and Western Europe. The diagnosis of LD and other TBDs faces significant obstacles due to the complex immune evasion strategies employed by pathogens, the inherent limitations of existing tests, and the challenge of detecting low levels of PCR template in human samples. However, advancements in LD diagnostics and the potential of phages as molecular markers and diagnostic tools for bacterial infections offer promising new approaches to overcome these challenges. Furthermore, the use of phages as a proxy for *Borrelia* presence in blood samples holds immense potential for detecting and monitoring *Borrelia* infections. As technology continues to evolve, it is crucial to develop more sensitive and accurate methods for detecting and monitoring TBDs, ultimately improving patient outcomes and public health worldwide.

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Data Availability Statement: The Ter-qPCR and Bmer-qPCR assay includes a set of oligonucleotide primers and Taqman® probes for *in vitro* quantitative detection of *B. burgdorferi s.*l. and *B. miyamotoi.* The Ter-qPCR primers and probes are described in the patent application Ref. P184103.EP.01/T. Other relevant data supporting the findings of the study are available in this article or from the corresponding author upon request.

Conflicts of Interest: The authors declare no conflict of interest.

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