Current issue date:	20 December 2023	Original approval(s):
Expiry date:	19 December 2026	ISO 13485 - 20 December 2017
Certificate identity number:	10563577	

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A Longitudinal Study of a Large Clinical Cohort of Patients with Lyme Disease and Tick-Borne Co-Infections Treated with Combination Antibiotics

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Abstract: The rising prevalence of tick-borne infections (TBIs) necessitates further attention. This study retrospectively investigated the types of TBIs, symptoms, and if combination antibiotics were helpful within a patient cohort at an infectious disease clinic in Ireland. In this chart audit of 301 individuals (184 female, 117 male) tested for TBIs, 140 (46.51%) had positive antibody responses for TBIs from an ELISA (enzyme-linked immunoassay) that was based on a modified two-tiered testing protocol. A total of 93 (66.43%) patients had positive antibody responses to one TBI: 83 (59.29%) for Borrelia, 7 (5.00%) for Rickettsia, and 1 (0.71%) each for either Babesia, Bartonella, or Ehrlichia. The remaining 47 (33.57%) patients were infected with multiple TBIs. These patients were treated with combination antibiotics and monitored at two subsequent follow-ups. Only 2 of 101 patients (1.98%) had discontinued treatment by the second follow-up. In the first follow-up with 118 patients, 70 (59.32%) reported pain and 48 (40.68%) had neurological symptoms. In the next follow-up of 101 patients, 41 (40.59%) had pain while 30 (29.70%) had neurological symptoms. There were statistically significant reductions in the incidence of pain (41.43%) and neurological (37.50%) symptoms between follow-ups. Thus, our study demonstrates that combination antibiotics effectively relieve TBI symptoms with good patient tolerance.

Keywords: Lyme disease; tick-borne infections; tick-borne co-infections; Lyme symptoms; Borrelia; Babesia; Bartonella; Ehrlichia; Rickettsia

Citation: Xi, D.; Thoma, A.; Rajput-Ray, M.; Madigan, A.; Avramovic, G.; Garg, K.; Gilbert, L.; Lambert, J.S. A Longitudinal Study of a Large Clinical Cohort of Patients with Lyme Disease and Tick-Borne Co-Infections Treated with Combination Antibiotics. *Microorganisms* **2023**, *11*, 2152. https://doi.org/10.3390/microorganisms11092152

Academic Editor: Pat Nuttall

Received: 15 August 2023
Revised: 22 August 2023
Accepted: 23 August 2023
Published: 24 August 2023

1. Introduction

Globally, tick-borne infections (TBIs) are increasingly recognized as an important cause of zoonotic diseases [1]. In fact, tick-derived pathogens contribute to the bulk of vector-borne infections in Europe, Asia, and the temperate regions of North America [2]. Global warming is a significant driver of tick population growth, enabling their migration to higher altitudes and latitudes [3–5]. With the exception of Lyme borreliosis, tick-borne diseases are sometimes overlooked among vector-borne diseases [1]. Complex vector–pathogen–host interactions make an estimation of the national incidence challenging [5–7]. Tick-borne diseases can be concentrated in rural or agricultural settings [5,7], which might not receive adequate public health attention [1]. With a broad spectrum of microorganisms within ticks [6,7], further research into TBIs is crucial to improve diagnosis, treatment, and eradication.

The focus of research has mainly centered around the *Borrelia burgdorferi sensu lato* complex, which causes Lyme disease; the most prevalent tick-borne disease world-wide [3,4]. Within the *B. burgdorferi sensu lato* complex, the main pathogenic species are *B. burgdorferi sensu stricto* and *B. mayonii* in North America, and *B. afzelii* and *B. garinii* in Europe and Asia [8]. More recent surveillance data from the US estimated that 476,000 patients were treated for Lyme disease annually from 2010 to 2018 [9]. Within Europe, a systematic review by Vandekerckhove and colleagues [10] discovered a rising trend in the national incidence of Lyme disease in Norway and Finland. In another study, three countries, Switzerland, Belgium, and the Netherlands, recorded a national incidence of more than 100 per 100,000 population per year [7]. Limited data are available from countries such as Ireland, Portugal, and Spain [7,10]. A retrospective cohort study by Forde and co-workers [11] from 2012 to 2016 estimated the incidence to be 1.15 per 100,000 population per year for those between the ages of 2 and 18 in Ireland. In Ireland, the national incidence of tick-borne illnesses, such as Lyme disease, can be hard to estimate, as only Lyme Neuroborreliosis is a notifiable disease [12]. There were four notifications in 2021 in Ireland and the national neuroborreliosis notification rate is 0.08 per 100,000 population [12].

Lyme disease symptoms can be categorized as early localized, early disseminated, or late disseminated [13]. Initial symptoms of Lyme borreliosis usually appear 2–30 days after a tick bite [5]. Constitutional symptoms such as fever, malaise, muscle and joint aches, and *erythema migrans* rash, are described in the early stages of the disease [2,4,7,8,13]. An *erythema migrans* rash is a circular or ovoid erythematous lesion with a central clearing that resembles a target sign. It develops on average 7 days after a tick bite, but studies have reported the appearance of *erythema migrans* from 1 to up to 36 days after a Borrelia infection [8,14]. While *erythema migrans* is a classic sign of Lyme disease, it is not seen in all patients [4,7,8]. The early-disseminated stage usually begins within days to weeks and can manifest as multiple *erythema migrans*, Lyme carditis, or neurological deficits [4,7,8,13]. Bannwarth syndrome, a meningoradiculoneuritis due to Lyme neuroborreliosis, is one of the most common disease manifestations after *erythema migrans* [8,15,16]. Lyme carditis can lead to complications like atrioventricular blocks, including third-degree heart blocks, which can be fatal if untreated [8,17]. Lyme arthritis is among the most common late symptoms of Lyme disease [8]. Another late manifestation of Lyme borreliosis is *acrodermatitis chronica atrophicans*, a bluish-red dermatological discoloration of the extremities that can lead to tissue atrophy if untreated [18].

The diagnosis of Lyme disease is aided by clinical manifestations, such as *erythema migrans* and a positive patient history of exposure to tick-endemic areas or tick bites [8]. Careful evaluation is advised, as several publications have found that only about 14–32% of patients in the US recalled receiving a tick bite, and some patients do not present with *erythema migrans* [4,7,8,14]. Serological testing with a standard or modified two-tier testing protocol can support a diagnosis. Standard two-tier testing involves an initial enzyme immunoassay and the subsequent utilization of Western blotting [8]. In the modified two-tier testing protocol, two enzyme immunoassays are used [8]. Both immunoassays need to be positive to support the diagnosis of Lyme disease [8]. The modified two-tier testing protocol is more sensitive at detecting early infections and less labor-intensive [8].

Ticks can concurrently carry other Borrelia subspecies or microbes in addition to *B. burgdorferi* [19–23]. Rickettsiosis, Ehrlichiosis, Babesiosis, and Bartonellosis are other notable TBIs [2,6]. An important consideration is that infections with these pathogens give rise to vague and non-specific symptoms, unlike *erythema migrans* with Lyme disease [6]. Clinical presentations cannot reliably distinguish co-infections from mono-infections or uninfected patients [24]. In the eastern United States, the majority of tick-borne co-infections are Lyme disease and human babesiosis, which can have confounding impacts on the disease course and severity [6,14,21,23]. Co-infections with both *B. burgdorferi* and *B. microti* can increase the duration and severity of Lyme disease in the early phase of illness [6]. *B. burgdorferi* and *B. microti* also have a synergistic relationship that causes the higher parasitemia of *B. microti* in mice [6].

Another study in Switzerland found co-infections of *B. burgdorferi* with the spotted fever group Rickettsiae [25]. These patients are more likely to present with non-specific symptoms, such as myalgia and fatigue. The authors recommended co-infections to be ruled out during diagnosis, especially in endemic areas [25].

The antibiotic treatment for Lyme disease is determined by multiple factors, such as age, antibiotic tolerance and hypersensitivity, the type of symptoms, and the presence of co-infections [8,26,27]. Doxycycline, amoxicillin, or cefuroxime are all recommended for the first-line treatment of Lyme disease [26,27]. However, using combination antibiotics to treat long-term Lyme disease symptoms is controversial [16,28]. Debilitating chronic symptoms, such as pain, fatigue, and neurological symptoms, can arise from a Lyme borreliosis infection [26,29,30]. One possible cause is persistent *B. burgdorferi* infection, as the bacteria possess immune-evasion mechanisms, such as hindering complement activation and phagocytosis [29], existing as metabolically inactive forms like round bodies, and bacterial biofilm creation [31]. Prolonged inflammation, autoimmunity, or permanent physiological damage from an infection are other proposed mechanisms for chronic symptoms [26,30]. Post-treatment Lyme disease syndrome has been used to describe the chronic symptoms that persist even with antibiotic treatment, and without clinical or laboratory evidence of infection [26,28]. The most widely debated hypothesis is "Chronic Lyme disease (CLD)", which shares many similarities with post-treatment Lyme disease syndrome. There are two categories proposed for CLD: untreated CLD (CLD-U) and previously treated (CLD-PT), where the latter demands that CLD symptoms remain present continuously or in a relapsing/remitting pattern for a period of six months or more after therapy [32]. To date, there is no consensus on the suitability and duration of antibiotic treatment for the chronic symptoms of Lyme disease [16,28].

Given the increasing global prevalence of tick-borne illnesses, further research could help improve the management of infections and co-infections. Currently, there is a lack of updated research on the incidence of different TBIs within Ireland. In this study, we aimed to investigate the types of TBIs and symptoms within a cohort of 301 patients from an Irish infectious disease clinic. We categorized the types of single and multiple tick-borne infections faced in this cohort. Secondly, we investigated the efficacy and safety of using prolonged combination antibiotics for relieving chronic symptoms in this cohort. We focused on the most common symptoms faced by this patient cohort: muscle and joint pain, fatigue, and neurological symptoms.

2. Materials and Methods

2.1. Study Objectives

The aims of this study are:

1. To investigate the types of TBIs within a patient cohort at an infectious disease outpatient clinic in Ireland.
2. To evaluate the efficacy and safety of using prolonged combination antibiotics for resolving chronic symptoms from TBIs in this patient cohort.

2.2. Patient Recruitment

All the patients who presented to an infectious disease outpatient clinic at The Mater Misericordiae Hospital, Eccles Street, Dublin 7, Ireland, who were to be evaluated for Lyme disease and co-infections, were offered participation in this study, following the inclusion and exclusion criteria. These patients exhibited "Lyme-like" symptoms, non-specific flu-like illness with clinical suspicion of tick-borne infections [33]. For instance, patients might recollect tick bites, have been exposed to tick-endemic areas, or have developed a bull's-eye rash. Below are the inclusion and exclusion criteria for the study.

Inclusion Criteria	Exclusion Criteria
1. Male and female patients, >16 years of age, with a documented positive clinical history of a "Lyme-like" illness [33]. 2. Be willing and able to provide written in-formed consent before study participation. 3. Be willing and able to comply with the study protocol. 4. Patients who have valid contact details.	1. Patients unable or unwilling to provide consent.

2.3. Serology Analysis

An ELISA platform was used to assess IgM and IgG antibody responses to Borrelia spp (*B. afzelii* and *B. garinii*), Borrelia persister forms, Babesia, Bartonella, Ehrlichia, and Rickettsia in this patient cohort using a modified two-tiered testing protocol. Serological testing was conducted using the TICKPLEX® test at ArminLabs GmbH in Augsburg, Germany. TICKPLEX® has the capability to assess IgM and IgG immune responses present in human serum samples against various species of *Borrelia burgdorferi sensu lato* in both spirochete and persistent forms, as well as against co-infections and opportunistic microbes. Specifically, TICKPLEX® encompasses *Borrelia burgdorferi sensu stricto, Borrelia afzelii*, and *Borrelia garinii* in their spirochete and persistent forms. It also covers other pathogens, like *Babesia microti, Bartonella henselae, Ehrlichia chaffeensis, Rickettsia akari*, Coxsackievirus, Epstein–Barr virus, Human parvovirus B19, *Mycoplasma fermentans,* and *Mycoplasma pneumoniae* [34]. The serological results were compiled and entered into an Excel spreadsheet for the handling of the data and analysis. We indicated if patients had positive, weakly positive, or negative antibody responses to the microorganisms. Using the serological data, we categorized patients into those with one TBI and those with multiple TBIs.

2.4. Patient Symptom Monitoring

During the initial visit (T0) to this infectious disease clinic, a patient history, clinical examination, and the necessary clinical investigations were conducted. Combination antibiotic treatments were given after the clinical consultation. Although our protocol scheduled follow-ups at 3 and 6 months, some appointments were rescheduled due to the COVID-19 pandemic.

A first assessment questionnaire (Appendix A) with 56 questions was administered to all 301 patients. The first 14 questions covered personal information, tick bites, and consultations before arrival at the clinic. Questions 15 to 47 were related to patient symptoms and were split into these 6 categories: skin, general well-being, cardiac, rheumatological, neurological, and psychological. As there is no validated symptom-monitoring questionnaire for TBIs and co-infections, the questions for each category were prepared using the current knowledge of common Lyme disease clinical manifestations [4,7,8,13,15,16]. Patients were also asked to rate their general state of health on a scale of 1 to 10, where a higher score signified better health. The remaining questions were based on blood tests and treatment to date, and included a free-response question allowing patients to list any further symptoms not covered in the questionnaire. The responses to this questionnaire served (T0) as the baseline for symptom monitoring.

Patients who returned to the clinic at two subsequent follow-up time points (T1 and T2) were given a follow-up visit questionnaire with 15 questions (Appendix B). In this part, we focused on the patient-reported perception of their general state of health and the incidence of the three most common symptoms in this cohort at T1 and T2. Again, respondents were asked to rate their well-being from 1 (poorly) to 10 (feeling very good). They were also asked to list their most distressing symptoms.

2.5. Statistical Analysis of Symptom Severity

The results from the questionnaires were compiled and entered into an Excel spreadsheet for the handling of data. We utilized Python libraries, including SciPy [35], NumPy [36],

Pandas [37], Matplotlib [38], and Seaborn [39], to analyze and visualize symptom ratings at the T0, T1, and T2 time points. Pandas was used to organize and preprocess the symptom-rating data. NumPy allowed us to perform calculations and transformations on the data. Matplotlib was used to create visualizations, such as line plots and bar graphs. Seaborn provided specialized plots, like boxplots, to better understand the distribution and variability of the symptom ratings.

We employed a two-sample Kolmogorov–Smirnov (K-S) and Mann–Whitney U tests for the statistical analysis. The two-sample Kolmogorov–Smirnov test assessed the dissimilarity between two distributions, with the K-S statistic ranging from 0 to 1 [40]. A higher K-S statistic indicated a greater dissimilarity between the distributions. In addition to the Kolmogorov–Smirnov (K-S), we used Mann–Whitney U tests with a paired *t*-test and Cohen's *d* effect size to analyze the difference in symptom incidence between time points T1 and T2 [41–46].

The significance of the K-S, Mann–Whitney U, and paired *t*-test results were determined by evaluating the *p*-values. A *p*-value less than 0.05, 0.01, or 0.001 was considered significant, depending on the predefined significance level. A smaller *p*-value indicated stronger evidence against the null hypothesis, and suggested a significant difference between the distributions of symptom ratings at the different time points. Cohen's *d* effect size quantified the standardized difference between the means, and provided insights into the magnitude of the differences. Effect sizes of $d \geq 0.2$, $d \geq 0.5$, $d \geq 0.8$, and $d \geq 1$ were considered small, medium, large, and very large, respectively. This allowed us to evaluate the practical significance or strength of the observed differences between T1 and T2.

2.6. Patients' Antibiotic Tolerance

To assess the treatment response and tolerance, questions on antibiotic tolerance were asked and recorded in the follow-up questionnaires. Patients either continued with the antibiotics prescribed at T0, changed antibiotics, or discontinued antibiotics. These responses were labeled as A, B, and C, respectively, in the Excel spreadsheet that can be found at the link in the Supplementary Materials, Table S1. Clinical and laboratory tests such as renal and liver function tests were conducted at the initial visit and two follow-up visits to help assess patient tolerance. This information was entered into the same Excel spreadsheet (Supplementary Materials, Table S1). As the prolonged use of combination antibiotics can cause gut microbiome dysregulation, probiotics like kefir were also provided to the patients to help mitigate this.

2.7. Ethics Approval

This study received ethics approval from the Institutional Review Board of the Mater Misericordiae University Hospital (Institutional Review Board Reference: 1/378/1946). It complies with the study protocol (version 6), the EU CT Directive 2001/20/EC, GCP Commission Directive 2005/28/EC, ICH/GCP, the Declaration of Helsinki (1996 Version), and all other local and international applicable regulatory requirements.

3. Results

3.1. Patient Characteristics

A total of 301 patients, 184 (61.13%) females and 117 (38.87%) males, from ages 16 to 89 years old, presented to the infectious disease clinic over 15 months, from December 2019 to February 2022. Of the 301 patients who came to the clinic at T0, 227 (75.42%) resided in Ireland. Dublin was listed as the county of residence within Ireland for the highest number of patients (52 patients, 17.28%). For the other patients, they were from various counties, such as Kerry, Meath, Wexford, and Wicklow (Table 1). The remaining patient (0.33%) was from Aran Island. For the patients who resided outside of the Republic of Ireland, 68 (22.59%) came from the United Kingdom, 2 (0.66%) came from the United States, 1 (0.33%) came from Hungary, 1 (0.33%) came from New Zealand, and the remaining

patient (0.33%) came from Germany (Table 1). One (0.33%) patient did not indicate their country of residence.

Table 1. Patient characteristics.

Patient Characteristics	Number of Patients, *n* (Percentage)
Gender	
Male	117 (38.87)
Female	184 (61.13)
Place of Residence in Ireland	227 (75.42)
Aran Island	1 (0.33)
Carlow	3 (1.00)
Cavan	4 (1.33)
Clare	13 (4.32)
Cork	13 (4.32)
Donegal	13 (4.32)
Dublin	52 (17.28)
Fermanagh	3 (1.00)
Galway	10 (3.32)
Kerry	15 (4.98)
Kildare	1 (0.33)
Kilkenny	3 (1.00)
Laois	7 (2.33)
Leitrim	5 (1.66)
Limerick	4 (1.33)
Longford	4 (1.33)
Louth	7 (2.33)
Mayo	7 (2.33)
Meath	11 (3.65)
Monaghan	2 (0.66)
Offaly	3 (1.00)
Roscommon	3 (1.00)
Sligo	5 (1.66)
Tipperary	4 (1.33)
Waterford	6 (1.99)
Westmeath	6 (1.99)
Wexford	12 (3.99)
Wicklow	10 (3.32)
Place of Residence Outside Ireland	73 (24.25)
United Kingdom	68 (22.59)
United States	2 (0.66)
Hungary	1 (0.33)
Germany	1 (0.33)
New Zealand	1 (0.33)

3.2. Patient Cohort's Serology Results

Out of 301 patients, 140 patients (46.51%) were antibody-positive to TBI (Table 2), of which 93 (66.43%) were positive to one type of TBI. Of the positive cases, 83 individuals (59.29%) were solely infected with Borrelia, 7 individuals (5.00%) were antibody-positive for Rickettsia alone, and 3 individuals were infected solely with Babesia, Bartonella, or Ehrlichia (0.71% each) (Table 2). The remaining 47 patients (33.57%) were infected with multiple TBIs.

Table 2. The number of antibody-positive patients with single TBI.

Types of TBIs	Number of Antibody-Positive Patients, *n* (Percentage)
Borrelia	83 (59.29)
Rickettsia	7 (5.00)
Babesia	1 (0.71)
Bartonella	1 (0.71)
Ehrlichia	1 (0.71)

A total of 42 individuals (30.00%) had antibodies to Borrelia and co-infections with Babesia, Bartonella, Ehrlichia, or Rickettsia (Table 3). There were two (1.43%) individuals

with Babesia and Rickettsia co-infections, and two were infected with either Bartonella and Rickettsia (0.71%), or Bartonella and Babesia (0.71%). One patient (0.71%) was antibody-positive for Babesia, Rickettsia, and Ehrlichia.

Table 3. The number of antibody-positive patients with multiple TBIs.

Types of TBIs	Number of Antibody-Positive Patients, *n* (Percentage)
Borrelia combined with Babesia, Bartonella, Ehrlichia, or Rickettsia	42 (30.00)
Babesia and Rickettsia	2 (1.43)
Babesia, Rickettsia, and Ehrlichia	1 (0.71)
Bartonella and Rickettsia	1 (0.71)
Bartonella and Babesia	1 (0.71)

3.3. Tick Bites and Erythema Migrans

From the questionnaire, 73 (52.14%) patients who were antibody-positive recalled receiving a tick bite. Additionally, 65 patients (46.43%) did not experience a bull's-eye rash, 40 (28.57%) confirmed developing a rash, and 31 (22.14%) were unsure.

3.4. Analysis of Symptom Severity at T0, T1, and T2

Of the 140 antibody-positive patients, 118 returned at the T1 follow-up and 101 returned at the T2 follow-up. The patients who did not return for the follow-ups either experienced symptom resolution or missed their appointments.

The patients exhibited significant improvements in their health status during the follow-up visits at time points T1 and T2, compared to the baseline measurement at time point T0 (Figure 1). Three graphical representations were employed to comprehensively understand the symptom-rating distribution (Figure 1). These graphical and statistical analyses collectively reinforce the evidence of significant health improvements observed in patients throughout their follow-up visits.

Firstly, Figure 1A illustrates a histogram with kernel density estimation depicting the distribution of symptom ratings at time points T0, T1, and T2. Using a scale from 1 to 10, where 1 is feeling very low or poorly and 10 is feeling very good, we saw a right shift of the symptom-rating distribution curve at T1 and T2 as compared to T0. There was a further shift to the right of the distribution curve from T1 to T2. At T0, the distribution curve peaked around scores 2–3, while the T1 distribution curve peaked at 5. The distribution curve for T2 has a plateau around scores 5–6, with the highest peak at score 7.

Secondly, Figure 1B shows the cumulative probability distribution, which offers insights into the overall distribution and relative probabilities of the observed symptom ratings at the three time points. To quantitatively assess the dissimilarity between the distributions at the three time points, a two-sample Kolmogorov–Smirnov (K-S) test was conducted, and the resulting p-value of 0.001 was used to determine the significance of this dissimilarity. There were statistically significant differences in the distribution of symptom ratings from T0 to T2 (K-S statistic = 0.65, $p \leq 0.001$), T0 to T1 (K-S statistic = 0.45, $p \leq 0.001$), and T1 to T2 (K-S statistic = 0.32, $p \leq 0.001$). There was a greater difference in the distribution of symptom ratings from T0 to T1 than from T1 to T2.

Lastly, a boxplot (Figure 1C) is utilized to illustrate the increase in median symptom ratings from T0 to T2, T0 to T1, and T0 to T2. The median symptom ratings were approximately 3, 5, and 6 for T0, T1, and T2, respectively. A Mann–Whitney U test assessed the differences between the T0, T1, and T2 time points. There were statistically significant improvements in the median symptom ratings from T0 to T1 (U = 2918.50, $p \leq 0.001$) and T2 (U = 1541.00, $p \leq 0.001$) and from T1 to T2 (U = 4068.50, $p \leq 0.001$).

3.5. Analysis of Chronic Persisting Symptoms

From the analysis of the questionnaire results, the three most common symptoms reported by the patients were pain, fatigue, and neurological symptoms, such as a tingling

sensation in the limbs and memory defects. At the first follow-up at T1, out of 118 patients, 70 (59.32%) patients experienced pain, 48 (40.68%) reported neurological symptoms, and 57 (48.31%) had fatigue (Table 4).

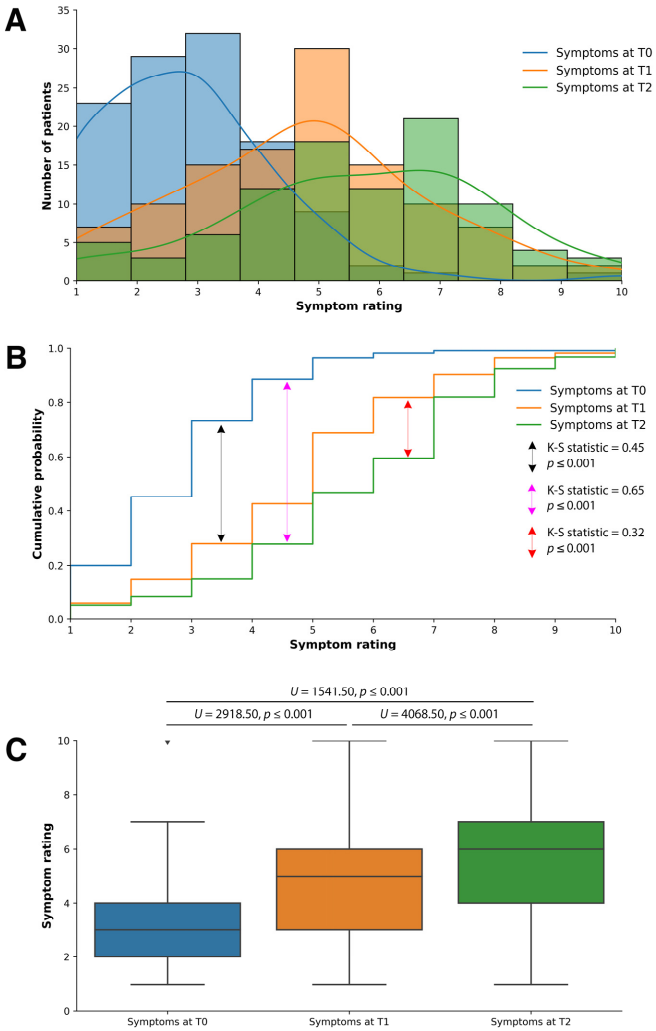

Figure 1. The patients' overall health saw significant statistical changes from T0 to T2. (**A**) A histogram with kernel density estimation depicting the distribution of symptom ratings at time points T0, T1, and T2. (**B**) The cumulative probability distribution of the overall distribution and relative probabilities of the observed symptom ratings at the three time points. (**C**) A boxplot illustrates the median symptom-ratings increase from T0 to T1 to T2.

Table 4. Analysis of the incidence of pain, fatigue, and neurological symptoms between T1 and T2.

	Pain	Neurological	Fatigue
Patients affected at T1 (*n*)	70	48	57
Patients affected at T2 (*n*)	41	30	47
Overall decrease in affected patients (%)	41.43	37.50	17.54
Paired *t*-test (*p* value)	<0.001	<0.01	>0.05
Cohen's *d* (95% CI)	0.43 (0.32–0.53)	0.28 (0.18–0.38)	0.15 (0.04–0.25)
Cohen's *d* interpretation	Medium	Medium	Small

Some patients' symptoms had improved by the second follow-up (T2). Of the 101 patients who returned to the clinic at both T1 and T2, 41 (40.59%) patients were still

suffering from pain, while neurological symptoms persisted in 30 (29.70%) patients, and 47 (46.53%) patients reported fatigue (Table 4).

The number of patients suffering from pain, neurological symptoms, and fatigue decreased by 41.43%, 37.50%, and 17.54%, respectively (Table 4). A significant statistical difference in pain and neurological symptoms between T1 and T2 was noticed, with a medium Cohen's *d* effect size (Table 4). For fatigue, the difference in incidence between T1 and T2 was not statistically significant, and a small Cohen's *d* effect size was observed (Table 4).

3.6. Antibiotic Treatment and Tolerance in Antibody-Positive Patients at T2

Among the 101 antibody-positive patients who returned for both T1 and T2 follow-ups, 95 (94.06%) patients were given a triple antibiotic combination regimen to be taken twice daily (Table 5). Most, 76 (72.65%) patients out of 101, were treated with a triple antibiotic regimen of 500 mg cefuroxime, 300 mg rifampicin, and 300 mg lymecycline. A total of six (5.94%) patients were given two antibiotic combination regimens (Table 5). A table summary of the number of patients prescribed with each type of combination antibiotic regimen is shown below (Table 5).

Table 5. The number of patients prescribed with each type of combination antibiotic regimen.

Antibiotic Combination	Number of Patients, *n* (Percentage)
500 mg cefuroxime, 300 mg rifampicin and 300 mg lymecycline	76 (75.25)
500 mg cefuroxime, 300 mg rifampicin and 500 mg azithromycin	6 (5.94)
500 mg cefuroxime, 300 mg rifampicin and 500 mg clarithromycin	4 (3.96)
300 mg rifampicin, 300 mg lymecycline and 500 mg azithromycin	7 (6.93)
300 mg rifampicin, 300 mg lymecycline and 500 mg clarithromycin	1 (0.99)
1000 mg cefuroxime, 300 mg rifampicin and 300 mg lymecycline	1 (0.99)
500 mg cefuroxime and 300 mg rifampicin	4 (3.96)
300 mg rifampicin and 300 mg lymecycline	1 (0.99)
300 mg rifampicin and 500 mg azithromycin	1 (0.99)

The duration of antibiotic treatment from T0 to T2 ranged from 12 weeks to 40 weeks. From the questionnaire responses, 77 of the 101 patients who returned for both follow-ups (76.24%) indicated that they still tolerated the antibiotic treatment (Supplementary Materials, Table S1). Due to side effects, 12 (11.88%) patients required a change in the antibiotic combination and 2 (1.98%) discontinued treatment. The remaining 10 patients did not provide an answer about antibiotic tolerance in the questionnaire at T2. Complete information on antibiotic duration, antibiotic tolerance, renal function tests, and liver function tests can be found in the supplementary materials (Supplementary Materials, Table S1).

One participant stopped the antibiotic treatment at 32 weeks but did not indicate the reason for discontinuation in the questionnaire. When asked at the first follow-up, she had previously tolerated the antibiotic treatment at 8 weeks. Her second renal and liver profile investigations showed that her CO2 total (31 mmol/L) and bilirubin (4 µmol) were outside the reference range of 22–29 mmol/L and 5–24 µmol, respectively (Supplementary Materials, Table S1). Her third liver profile showed an AST value of 17 I.U./L, which was lower than the reference range of 19–42 I.U./L. In isolation, these findings have an unclear clinical significance.

The last patient who ceased antibiotic treatment stopped at 16 weeks. She answered in her questionnaire that it was due to severe pain under her ribs that required a morphine injection. She restarted the antibiotic regimen at a lower dose 10 days later. She tolerated

the antibiotic regimen at her first follow-up at 8 weeks. Her renal and liver function tests performed at 19 weeks did not show a significant deviation from her baseline at the initial visit, although her CO2 total was higher than the reference values, at 32 mmol/L (Supplementary Materials, Table S1).

4. Discussion

Our findings (Tables 2 and 3) support the notion that infection from the *Borrelia burgdorferi* species is the most predominant TBI in Ireland, with most of the antibody-positive cases (59.29%) in this cohort being solely infected with Borrelia. A total of 42 out of 140 patients (30.00%) had co-infections of Borrelia with other TBIs, such as Babesia, Bartonella, Ehrlichia, and Rickettsia. This is notable, as earlier publications have established that co-infections with Ehrlichiosis and Babesiosis can complicate the disease course and treatment [6,14,21,23]. Furthermore, past research has also noted that *B. burgdorferi* can cause immune dysfunction and hinder the development of IgG-producing plasma cells [47]. One study also demonstrated that *B. burgdorferi* has immunosuppressive effects, as mice who were infected had less capability to produce antibodies against influenza [47]. Immune system derangements in TBIs could also impact the pathogenesis of tick-borne co-infections, as seen in the synergistic relationship between *B. burgdorferi* and *B. microti* co-infections, which cause higher serum levels of *B. microti* in mice [6]. It is important to consider and test for co-infections, especially in endemic areas and for those with unusual non-specific symptoms, or abnormal investigation results [25,27].

From our first assessment questionnaire results, only 52.14% of all the antibody-positive patients recalled receiving a tick bite. Some publications from the US found that only about 14–32% of patients recalled a tick bite [14]. Additionally, our questionnaire showed that 46.43% of the patients did not experience a bull's-eye rash, and 22.14% were unsure if they had developed a rash. Only 28.57% of all the antibody-positive patients could confirm they had a rash. From the past literature, *erythema migrans* are not seen in all patients [4,7,8]. Our study thus highlights the importance of not relying solely on a positive tick bite or positive *erythema migrans* to consider a Lyme disease diagnosis.

Based on this patient cohort, we believe there is merit in using prolonged combination antibiotics to relieve the lingering symptoms from TBIs. For this patient cohort, the three most commonly reported patient symptoms were pain, fatigue, and neurological symptoms, such as a tingling sensation in the limbs and memory defects. These three symptoms were among the most reported persisting symptoms by others [26,30]. A total of 94.06% of the patients who returned to the clinic at both T1 and T2 had been prescribed three antibiotics, and the remaining 5.94% were given two antibiotics from T0 to T2. Although current guidelines by the IDSA (Infectious Diseases Society of America) and ILADS (International Lyme and Associated Diseases Society) sometimes differ on the optimum duration of antibiotic treatment, both do not recommend treatment beyond 6 weeks without clinical reassessment [27,48]. These guidelines also recommended single antibiotic treatment for Lyme disease in most circumstances [27,48]. Earlier studies were inconclusive for determining the efficacy of long-term combination antibiotics [16,28]. However, our study illustrated that treatment with prolonged combination antibiotics is effective and has a good safety profile (Supplementary Materials, Table S1). From the results of our questionnaire, many patients had a general improvement in symptom severity from T0 to T1 and subsequently from T1 to T2. We also demonstrate a statistically significant difference in the incidence of pain and neurological symptoms between T1 and T2. Most antibody-positive patients who returned for both follow-ups tolerated the prolonged use of combination antibiotics, and only two (1.98%) discontinued the antibiotic treatment. Other publications have also found combination antibiotics effective in clearing persister forms of *B. burgdorferi* [49]. Current guidelines should consider prolonged combination antibiotics as a treatment for Lyme disease and co-infections.

This study was a retrospective analysis of patients presenting in a clinical setting who were prescribed, on a case-by-case basis, an antibiotic regimen. With close monitoring,

the individuals were assessed with regards to antibiotic tolerability, allergies, safety, and potential efficacy. As this was a preliminary study to highlight treatment safety and the improvement in patient well-being and symptoms, further research should be conducted to find the most effective combination antibiotic regimen for the various clinical manifestations of Lyme disease.

We discovered no statistically significant difference in the incidence of fatigue between T1 and T2. An earlier randomized controlled trial of 55 patients with severe fatigue 6 months after antibiotic treatment for Lyme disease by Krupp and colleagues [50] showed that IV ceftriaxone for 28 days improved symptoms. In our study, we used the incidence of fatigue, instead of assessing the reduction in fatigue severity, with a 11-item questionnaire like Krupp and colleagues [50]. Another difference is the route of administration, as patients were managed in an outpatient setting and were not given IV antibiotics. This could mean that fatigue is a chronic symptom of Lyme disease that requires specific management and a more sensitive assessment tool to monitor treatment effect.

A limitation of this study is the lack of validated patient-reported symptom questionnaire specific for Lyme disease or other tick-borne infections. Our questionnaires were created based on the existing research literature on the common clinical manifestations of Lyme disease and the clinical experience of specialists in this area. Using the questionnaires to monitor patient-reported symptoms, our study assessed the most important clinical symptoms in our patient cohort.

5. Conclusions

Our study established that most patients in this cohort were infected with the *Borrelia burgdorferi* species, and about a third had co-infections with other tick-borne pathogens. Approximately half of the patients recalled receiving a tick bite and developing a bull's-eye rash. Pain, fatigue, and neurological symptoms were among the most common persistent symptoms faced by this cohort from the initial visit to T2. With the use of long-term combination antibiotics, we noted symptom resolution from the initial visit to T2 with good patient tolerance.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms11092152/s1, Table S1: Data for antibiotic tolerance, renal and liver function tests.

Author Contributions: Conceptualization, A.T., M.R.-R., A.M., G.A., L.G. and J.S.L.; methodology, A.T., M.R.-R., G.A., K.G., L.G. and J.S.L.; software, K.G.; formal analysis, D.X., A.T., M.R.-R., A.M., G.A., K.G., L.G. and J.S.L.; investigation, D.X., A.T., M.R.-R., A.M., G.A., K.G., L.G. and J.S.L.; resources, M.R.-R., A.M., G.A., L.G. and J.S.L.; data curation, D.X., A.T., M.R.-R., A.M., G.A., K.G., L.G. and J.S.L.; writing—original draft preparation, D.X.; writing—review and editing, M.R.-R., G.A., K.G., L.G. and J.S.L.; visualization, D.X., A.T. and K.G.; supervision, M.R.-R., G.A., L.G. and J.S.L.; project administration, G.A., L.G. and J.S.L.; funding acquisition, J.S.L. All authors have read and agreed to the published version of the manuscript.

Funding: The Mater Foundation granted some funding towards the analysis of this research which was otherwise funded by a donation from a patient.

Data Availability Statement: All of the relevant data are provided in the form of regular figures, tables, and Supplementary Materials file.

Acknowledgments: The authors extend their appreciation to the patient who made a generous donation to our research. We would also like to thank the Mater Foundation for the research grant and all patients involved in this study.

Conflicts of Interest: K.G. and L.G. have a financial and business interest in Te?ted Oy. There is no commercial partnership between Te?ted Oy. and the institutions of the other authors. K.G. and L.G. assisted in the analyses of the clinical research data but had no role in the collection of data. Other authors declare no conflicts of interest.

Appendix A

Symptoms Monitoring Check List

Patient Questionnaire: *First Assessment*

JL _____ ***DOB*** _____ ***MALE*** ◯ **Female** ◯ ***Date*** _____

Q1. If you reside in **Ireland** please state the **County that you live in** _____

Q2. If you reside **outside Ireland** please state where: _____

Q3. Do you engage in **outdoor hobbies and activities**? _____

Q4. Were you diagnosed previously with a **chronic illness?** eg. arthritis, heart problems, depression, psychiatric illness? **No** ◯ If yes please specify / list which illness(es).

_____

Q5. Do you **recall getting a tick bite**? **Yes** ◯ **No** ◯

Q6. If yes **how long ago**? Days _____ Months _____ Years _____

Q7. If yes do you **recall the location /area**? **No** ◯ **Yes** _____

Q8. If yes what is **the interval** between the bite and the onset of your symptoms?

Days _____ **Months** _____ **Years** _____ **don't remember** ◯

Q9. Did you **visit your GP** with these symptoms? **Yes** ◯ **No** ◯

If yes **were you satisfied** with the outcome of your visit? **Yes** ◯ **No** ◯

Q10. Did you visit **another consultant** with these symptoms? **Yes** ◯ **No** ◯

If yes were you satisfied with the outcome of your visit? **Yes** ◯ **No** ◯

Q11. How many doctor visits have you had to address your ongoing symptoms? _____

Q12. How would you rate the **treatment and the care** that you have received for your symptoms before today? (Please circle your response)

Poor 1 2 3 4 5 6 7 8 9 10 **Very Good**

Q13. What is your **current employment** status?

Working ◯ Unemployed ◯ Retired ◯ Caring responsibilities ◯ sick leave ◯ Other ◯

Q14. Have your **symptoms affected your employment status?** **Yes** ◯ **No** ◯

SKIN

Q15. Have you experienced a **red expanding circular rash**? (Bulls Eye)

Yes ◯ **No** ◯**Unsure** ◯

Q16. Have you experienced an **unexplained rash across your body**? **Yes** ◯ **No** ◯**Unsure** ◯

General Wellbeing

Q17. Have you experience **unexplained fevers/chills / sweats** in the last six months**?**

Yes ◯ **No** ◯

Q18. In the last six months have you been complaining of a **sore throat**? **Yes** ◯ **No** ◯

Q19. In the last six months are you experiencing **unexplained headaches?** **Yes** ◯ **No** ◯

Q20. Have you experienced **swollen glands** in the last six months? **Yes** ◯ **No** ◯ **Unsure** ◯

Q21. In the last six months are you experiencing **unexplained severe fatigue** not relieved by rest? **Yes** ◯ **No** ◯

Q22. If yes, how would you rate the level of **severe fatigue** that you have experienced in the last six months? Please circle your response below.

No fatigue 1 2 3 4 5 6 7 8 9 10 **Severe fatigue**

Q23. How would you rate **how you are feeling today** regarding your symptoms? (please circle your response below)

Poorly 1 2 3 4 5 6 7 8 9 10 **Very Well.**

Cardiac

Q24. Have you experienced **unexplained chest pain** in the last six months? **Yes** ◯ **No** ◯

Q25. Have you experienced **unexplained shortness of breath** in the last six months?

Yes ◯ **No** ◯

Q26. Have you experienced **unexplained heart palpitations** in the last six months?

Yes ◯ **No** ◯

Q27. When you stand up from sitting do you experience "lightheadedness" over the last six months? **Yes** ◯ **No** ◯

Rheumatological

Q28. Are you experiencing **unexplained pain in your joints** in the last six months?
Yes ◯ **No** ◯

Q29. If yes, does the pain **move to different joints?** **Yes** ◯ **No** ◯

Q30. If yes does the **intensity of the pain** change from day to day? **Yes** ◯ **No** ◯

Q31. Are you experiencing **unexplained swelling in your joints** in the last six months? **Yes** ◯ **No** ◯

Q32. Have you experienced **weakness in your muscles** in the last six months? **Yes** ◯ **No** ◯

Q33. Have you experienced **muscle pain** in the last six months? **Yes** ◯ **No** ◯

Q34. How would you rate the **pain in your joints or muscles** in the last six months?
(please circle your response below).

No Pain 1 2 3 4 5 6 7 8 9 10 **Severe Pain**

Neurological

Q35. Have your experienced tingling in your face / **facial paralysis?** (Bell's Palsy)
Yes ◯ **No** ◯

Q36. Have you experienced an unexplained **tingling sensation in your arms and hands** in the last six in months? **Yes** ◯ **No** ◯

Q37. Have you experienced **unexplained numbness in your arms and hands** in the last six months? **Yes** ◯ **No** ◯

Q38. Have you experienced **unexplained difficulty with your concentration** in the last six months? **Yes** ◯ **No** ◯

Q39. Have you experienced **unexplained sleep disturbance** in the last six months? **Yes** ◯ **No** ◯

Q40. Have you experienced **unexplained problems with your eyes / your vision** in the last six months? **Yes** ◯ **No** ◯

Q41. Have you experienced unexplained **neck stiffness** in the last six months? **Yes** ◯ **No** ◯

Q42. Have you experienced **unexplained tinnitus (ringing in your ears)** in the last six months? **Yes** ◯ **No** ◯

Psychological

Q43. Have you or your family noticed **changes in your personality** in the last six months?

Yes ◯ **No** ◯

Q44. Do you feel **"down" or in low mood** because of your symptoms? **Yes** ◯ **No** ◯

Q45. If yes how would you rate your **overall mood** because of your symptoms?

Feeling in low form 1 2 3 4 5 6 7 8 9 10 In very good form

a lot of the time most of the time

Q46. In the last six months have you experienced **bouts of anger** because of your symptoms?
Yes ◯ **No** ◯

Q47. In the last six months have you experienced **bouts of anxiety** because of your symptoms?
Yes ◯ **No** ◯

Blood Tests

Q48. Did you have any **blood tests done previously, specifically** to identify a lyme like illness?

No ◯ **Yes** _____

Q49. Where was your blood sample analysed?

(a) Germany ◯ (b) UK. ◯ Irish Test ◯ OTHER ◯

Treatment to Date

Q50. Have you been **prescribed antibiotic therapy previously** for your current symptoms?

Yes ◯ **No** ◯

Q51. If yes, can you recall which antibiotic was prescribed for you?

No ◯ **Yes** _____

Q52. If yes for **how long did you take the antibiotics**? _____

Q53. Did you experience any **improvement of your symptoms** on antibiotic treatment**?**

Yes ◯ **No** ◯

Q54. Have you resorted to using **other treatments** to relieve your symptoms eg.(a) Herbal treatment, (b)acupuncture,(c) homeopath, (d)detox treatments, etc. Please list and specify.

_____

_____

Q55. Have you found any of these treatments successful in relieving your symptoms?

Yes ◯ No ◯ Unsure ◯

If yes, please **specify / list the option** (s) that helped relieve your symptoms.

_____

Q56. Feel free to add any further **unexplained symptoms** that you are experiencing.

_____

Thank you for completing this assessment questionnaire

Appendix B

Follow Up Visit

JL:_____ Initials:_____ Date _____

Q1. Were you **diagnosed with a Lyme-like illness by Prof. Lambert?** **No** ◯ **Yes** ◯ **Date _____**

Q2. How long did you have **symptoms of this illness prior to your diagnosis**? **Months ___Years ___**

Q3. Were you prescribed **antibiotic therapy** at your first visit here? **Yes** ◯ **No** ◯

Q3a. If yes for **how long did you take antibiotic therapy in total?_____ weeks _____ months**

Q4. How did you tolerate the antibiotic/s. Please circle below

(a) No problem with antibiotics ◯ **(b) had to change antibiotic due to side effects** ◯
(c) discontinued the antibiotics due to side effects ◯

Q5. Did you take medication to boost your immune system? **Yes** ◯ **No** ◯

Q6. Did you take herbal treatment to help your symptoms? **Yes** ◯ **No** ◯

Q7. Do you still have symptoms of a Lyme – like illness? **Yes** ◯ **No** ◯

Q8. If yes **which symptom/s** is the most distressing for you? _____

Q9. In the months **prior to your diagnosis** how would you rate how you were feeling about your health?

Feeling very low 1 2 3 4 5 6 7 8 9 10 **Feeling very well**

Q10. How are you **feeling today regarding your lyme-like symptoms**

Poorly 1 2 3 4 5 6 7 8 9 10 **Feeling very well**

Q11. How would you rate your overall **pain level today in your joints / muscles?**

No Pain 1 2 3 4 5 6 7 8 9 10 **Severe Pain**

Q12. How would you rate your level of fatigue today?

No fatigue 1 2 3 4 5 6 7 8 9 10 **Severe fatigue**

Q13. How would you rate your overall mood today because of your symptoms?

Feeling in low mood 1 2 3 4 5 6 7 8 9 10 In Good Form

Q14. Prior to your diagnosis were you aware that **tick borne illnesses are prevalent in Ireland?**

Yes ◯**No** ◯

Q15. How would you rate the treatment and care that you have received for the past six months at the tickborne infections consultation service?

Not satisfied 1 2 3 4 5 6 7 8 9 10 **Very Satisfied**

Additional Comments _____

Thank you for completing this questionnaire

References

1. Lippi, C.A.; Ryan, S.J.; White, A.L.; Gaff, H.D.; Carlson, C.J. Trends and Opportunities in Tick-Borne Disease Geography. *J. Med. Entomol.* **2021**, *58*, 2021–2029. [CrossRef] [PubMed]
2. Rochlin, I.; Toledo, A. Emerging tick-borne pathogens of public health importance: A mini-review. *J. Med. Microbiol.* **2020**, *69*, 781–791. [CrossRef] [PubMed]
3. Gilbert, L. The Impacts of Climate Change on Ticks and Tick-Borne Disease Risk. *Annu. Rev. Entomol.* **2021**, *66*, 373–388. [CrossRef]
4. Boulanger, N.; Boyer, P.; Talagrand-Reboul, E.; Hansmann, Y. Ticks and tick-borne diseases. *Med. Mal. Infect.* **2019**, *49*, 87–97. [CrossRef]
5. Lindgren, E.; Jaenson, T.G.T. *Lyme Borreliosis in Europe: Influences of Climate and Climate CHANGE, Epidemiology, Ecology and Adaptation Measures*; World Health Organisation, Regional Office for Europe: Copenhagen, Denmark, 2006.
6. Diuk-Wasser, M.A.; Vannier, E.; Krause, P.J. Coinfection by Ixodes Tick-Borne Pathogens: Ecological, Epidemiological, and Clinical Consequences. *Trends Parasitol.* **2016**, *32*, 30–42. [CrossRef]
7. Burn, L.; Vyse, A.; Pilz, A.; Tran, T.M.P.; Fletcher, M.A.; Angulo, F.J.; Gessner, B.D.; Moisi, J.C.; Stark, J.H. Incidence of Lyme Borreliosis in Europe: A Systematic Review (2005–2020). *Vector Borne Zoonotic Dis.* **2023**, *23*, 172–194. [CrossRef]
8. Madison-Antenucci, S.; Kramer, L.D.; Gebhardt, L.L.; Kauffman, E. Emerging Tick-Borne Diseases. *Clin. Microbiol. Rev.* **2020**, *33*, e00083-18. [CrossRef]
9. Kugeler, K.J.; Schwartz, A.M.; Delorey, M.J.; Mead, P.S.; Hinckley, A.F. Estimating the Frequency of Lyme Disease Diagnoses, United States, 2010–2018. *Emerg. Infect. Dis.* **2021**, *27*, 616–619. [CrossRef] [PubMed]
10. Vandekerckhove, O.; De Buck, E.; Van Wijngaerden, E. Lyme disease in Western Europe: An emerging problem? A systematic review. *Acta Clin. Belg.* **2021**, *76*, 244–252. [CrossRef]
11. Forde, K.M.; O'Gorman, J.; Gavin, P.J.; Dryden, M.S.; Keady, D.; Hanahoe, B.; McDonnell, C.; Power, L.; Cryan, B.; Sweeney, J.; et al. The clinical presentation, treatment and outcome of serologically confirmed paediatric Lyme disease in the Republic of Ireland over a 5-year period: A retrospective cohort study. *Eur. J. Clin. Microbiol. Infect. Dis.* **2021**, *40*, 725–734. [CrossRef]
12. Health Protection Surveillance Centre. Lyme Disease. Available online: https://www.hpsc.ie/a-z/vectorborne/lymedisease/ (accessed on 14 August 2023).
13. Wong, K.H.; Shapiro, E.D.; Soffer, G.K. A Review of Post-treatment Lyme Disease Syndrome and Chronic Lyme Disease for the Practicing Immunologist. *Clin. Rev. Allergy Immunol.* **2022**, *62*, 264–271. [CrossRef] [PubMed]
14. Nadelman, R.B. Erythema migrans. *Infect. Dis. Clin. N. Am.* **2015**, *29*, 211–239. [CrossRef] [PubMed]
15. Garkowski, A.; Zajkowska, J.; Zajkowska, A.; Kulakowska, A.; Zajkowska, O.; Kubas, B.; Jurgilewicz, D.; Hladunski, M.; Lebkowska, U. Cerebrovascular Manifestations of Lyme Neuroborreliosis-A Systematic Review of Published Cases. *Front. Neurol.* **2017**, *8*, 146. [CrossRef] [PubMed]
16. Rauer, S.; Kastenbauer, S.; Fingerle, V.; Hunfeld, K.P.; Huppertz, H.I.; Dersch, R. Lyme Neuroborreliosis. *Dtsch. Arztebl. Int.* **2018**, *115*, 751–756. [CrossRef]
17. Forrester, J.D.; Mead, P. Third-degree heart block associated with lyme carditis: Review of published cases. *Clin. Infect. Dis.* **2014**, *59*, 996–1000. [CrossRef]
18. Gade, A.; Matin, T.; Rubenstein, R.; Robinson, C.A. *Acrodermatitis Chronica Atrophicans*; StatPearls: Treasure Island, FL, USA, 2023.
19. Raileanu, C.; Moutailler, S.; Pavel, I.; Porea, D.; Mihalca, A.D.; Savuta, G.; Vayssier-Taussat, M. Borrelia Diversity and Co-infection with Other Tick Borne Pathogens in Ticks. *Front. Cell. Infect. Microbiol.* **2017**, *7*, 36. [CrossRef]
20. Skotarczak, B.; Rymaszewska, A.; Wodecka, B.; Sawczuk, M. Molecular evidence of coinfection of *Borrelia burgdorferi* sensu lato, human granulocytic ehrlichiosis agent, and *Babesia microti* in ticks from northwestern Poland. *J. Parasitol.* **2003**, *89*, 194–196. [CrossRef]
21. Lou, Y.; Liu, L.; Gao, D. Modeling co-infection of Ixodes tick-borne pathogens. *Math. Biosci. Eng.* **2017**, *14*, 1301–1316. [CrossRef]
22. Cutler, S.J.; Vayssier-Taussat, M.; Estrada-Pena, A.; Potkonjak, A.; Mihalca, A.D.; Zeller, H. Tick-borne diseases and co-infection: Current considerations. *Ticks Tick Borne Dis.* **2021**, *12*, 101607. [CrossRef]
23. Swanson, S.J.; Neitzel, D.; Reed, K.D.; Belongia, E.A. Coinfections acquired from ixodes ticks. *Clin. Microbiol. Rev.* **2006**, *19*, 708–727. [CrossRef]
24. Dunaj, J.; Moniuszko-Malinowska, A.; Swiecicka, I.; Andersson, M.; Czupryna, P.; Rutkowski, K.; Zambrowski, G.; Zajkowska, J.; Grygorczuk, S.; Kondrusik, M.; et al. Tick-borne infections and co-infections in patients with non-specific symptoms in Poland. *Adv. Med. Sci.* **2018**, *63*, 167–172. [CrossRef] [PubMed]
25. Sandelin, L.L.; Askling, H.H. Tick-borne co-infections in Europe: Clinical conundrums. *New Microbes New Infect.* **2023**, *53*, 101157. [CrossRef] [PubMed]
26. Kullberg, B.J.; Vrijmoeth, H.D.; van de Schoor, F.; Hovius, J.W. Lyme borreliosis: Diagnosis and management. *BMJ* **2020**, *369*, m1041. [CrossRef] [PubMed]
27. Lantos, P.M.; Rumbaugh, J.; Bockenstedt, L.K.; Falck-Ytter, Y.T.; Aguero-Rosenfeld, M.E.; Auwaerter, P.G.; Baldwin, K.; Bannuru, R.R.; Belani, K.K.; Bowie, W.R.; et al. Clinical Practice Guidelines by the Infectious Diseases Society of America (IDSA), American Academy of Neurology (AAN), and American College of Rheumatology (ACR): 2020 Guidelines for the Prevention, Diagnosis and Treatment of Lyme Disease. *Clin. Infect. Dis.* **2021**, *72*, e1–e48. [CrossRef] [PubMed]

28. Garcia-Monco, J.C.; Benach, J.L. Lyme Neuroborreliosis: Clinical Outcomes, Controversy, Pathogenesis, and Polymicrobial Infections. *Ann. Neurol.* **2019**, *85*, 21–31. [CrossRef] [PubMed]
29. Borgermans, L.; Goderis, G.; Vandevoorde, J.; Devroey, D. Relevance of chronic lyme disease to family medicine as a complex multidimensional chronic disease construct: A systematic review. *Int. J. Family Med.* **2014**, *2014*, 138016. [CrossRef]
30. Dersch, R.; Hottenrott, T.; Schmidt, S.; Sommer, H.; Huppertz, H.I.; Rauer, S.; Meerpohl, J.J. Efficacy and safety of pharmacological treatments for Lyme neuroborreliosis in children: A systematic review. *BMC Neurol.* **2016**, *16*, 189. [CrossRef]
31. Rudenko, N.; Golovchenko, M.; Kybicova, K.; Vancova, M. Metamorphoses of Lyme disease spirochetes: Phenomenon of Borrelia persisters. *Parasit. Vectors* **2019**, *12*, 237. [CrossRef]
32. Shor, S.; Green, C.; Szantyr, B.; Phillips, S.; Liegner, K.; Burrascano, J.J., Jr.; Bransfield, R.; Maloney, E.L. Chronic Lyme Disease: An Evidence-Based Definition by the ILADS Working Group. *Antibiotics* **2019**, *8*, 269. [CrossRef]
33. Aucott, J.N.; Seifter, A. Misdiagnosis of early Lyme disease as the summer flu. *Orthop. Rev.* **2011**, *3*, e14. [CrossRef]
34. Garg, K.; Jokiranta, T.S.; Filen, S.; Gilbert, L. Assessing the Need for Multiplex and Multifunctional Tick-Borne Disease Test in Routine Clinical Laboratory Samples from Lyme Disease and Febrile Patients with a History of a Tick Bite. *Trop. Med. Infect. Dis.* **2021**, *6*, 38. [CrossRef]
35. Virtanen, P.; Gommers, R.; Oliphant, T.E.; Haberland, M.; Reddy, T.; Cournapeau, D.; Burovski, E.; Peterson, P.; Weckesser, W.; Bright, J.; et al. SciPy 1.0: Fundamental algorithms for scientific computing in Python. *Nat. Methods* **2020**, *17*, 261–272. [CrossRef]
36. Harris, C.R.; Millman, K.J.; van der Walt, S.J.; Gommers, R.; Virtanen, P.; Cournapeau, D.; Wieser, E.; Taylor, J.; Berg, S.; Smith, N.J.; et al. Array programming with NumPy. *Nature* **2020**, *585*, 357–362. [CrossRef]
37. The Pandas Development Team. pandas-dev/pandas: Pandas. Zenodo, 2023. Available online: https://zenodo.org/record/8239932 (accessed on 14 August 2023).
38. Hunter, J.D. Matplotlib: A 2D graphics environment. *Comput. Sci. Eng.* **2007**, *9*, 90–95. [CrossRef]
39. Waskom, M.L. Seaborn: Statistical data visualization. *J. Open Source Softw.* **2021**, *6*, 3021. [CrossRef]
40. Hodges, J.L.J. The significance probability of the Smirnov two-sample test. *Arkiv. Fiur Matematik.* **1957**, *3*, 469–486. [CrossRef]
41. Mann, H.B.; Whitney, D.R. On a test of whether one of two random variables is stochastically larger than the other. *Ann. Math. Stat.* **1947**, *18*, 50–60. [CrossRef]
42. Fay, M.P.; Proschan, M.A. Wilcoxon-Mann-Whitney or *t*-test? On assumptions for hypothesis tests and multiple interpretations of decision rules. *Stat. Surv.* **2010**, *4*, 1–39. [CrossRef]
43. Lakens, D. Calculating and reporting effect sizes to facilitate cumulative science: A practical primer for t-tests and ANOVAs. *Front. Psychol.* **2013**, *4*, 863. [CrossRef]
44. Di Bucchianico, A. Combinatorics, computer algebra, and the Wilcoxon-Mann-Whitney test. *J. Stat. Plan. Inference* **1999**, *79*, 349–364. [CrossRef]
45. Sullivan, G.M.; Feinn, R. Using Effect Size-or Why the P Value Is Not Enough. *J. Grad. Med. Educ.* **2012**, *4*, 279–282. [CrossRef]
46. Hedges, L.; Olkin, I. *Statistical Methods for Meta-Analysis*; Academic Press: San Diego, CA, USA, 1985.
47. Sellati, T.J.; Barberio, D.M. Mechanisms of Dysregulated Antibody Response in Lyme Disease. *Front. Cell Infect. Microbiol.* **2020**, *10*, 567252. [CrossRef]
48. Cameron, D.J.; Johnson, L.B.; Maloney, E.L. Evidence assessments and guideline recommendations in Lyme disease: The clinical management of known tick bites, erythema migrans rashes and persistent disease. *Expert. Rev. Anti. Infect. Ther.* **2014**, *12*, 1103–1135. [CrossRef]
49. Feng, J.; Li, T.; Yee, R.; Yuan, Y.; Bai, C.; Cai, M.; Shi, W.; Embers, M.; Brayton, C.; Saeki, H.; et al. Stationary phase persister/biofilm microcolony of Borrelia burgdorferi causes more severe disease in a mouse model of Lyme arthritis: Implications for understanding persistence, Post-treatment Lyme Disease Syndrome (PTLDS), and treatment failure. *Discov. Med.* **2019**, *27*, 125–138.
50. Krupp, L.B. Fatigue in multiple sclerosis: Definition, pathophysiology and treatment. *CNS Drugs* **2003**, *17*, 225–234. [CrossRef]

Tropical Medicine and Infectious Disease

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Assessing the Need for Multiplex and Multifunctional Tick-Borne Disease Test in Routine Clinical Laboratory Samples from Lyme Disease and Febrile Patients with a History of a Tick Bite

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Abstract: Human polymicrobial infections in tick-borne disease (TBD) patients is an emerging public health theme. However, the requirement for holistic TBD tests in routine clinical laboratories is ambiguous. TICKPLEX® PLUS is a holistic TBD test utilized herein to assess the need for multiplex and multifunctional diagnostic tools in a routine clinical laboratory. The study involved 150 specimens categorized into Lyme disease (LD)-positive (n = 48), LD-negative (n = 30), and febrile patients from whom borrelia serology was requested (n = 72, later "febrile patients") based on reference test results from United Medix, Finland. Reference tests from DiaSorin, Immunetics, and Mikrogen Diagnostik followed the two-tier LD testing system. A comparison between the reference tests and TICKPLEX® PLUS produced 86%, 88%, and 87% positive, negative, and overall agreement, respectively. Additionally, up to 15% of LD and 11% of febrile patients responded to TBD related coinfections and opportunistic microbes. The results demonstrated that one (TICKPLEX® PLUS) test can aid in a LD diagnosis instead of four tests. Moreover, TBD is not limited to just LD, as the specimens produced immune responses to several TBD microbes. Lastly, the study indicated that the screening of febrile patients for TBDs could be a missed opportunity at reducing unreported patient cases.

Keywords: Lyme disease; tick-borne disease; zoonoses; spirochetes; polymicrobial; summer flu; misdiagnosis; persister; Borrelia; Lyme diagnostic

Citation: Garg, K.; Jokiranta, T.S.; Filén, S.; Gilbert, L. Assessing the Need for Multiplex and Multifunctional Tick-Borne Disease Test in Routine Clinical Laboratory Samples from Lyme Disease and Febrile Patients with a History of a Tick Bite. *Trop. Med. Infect. Dis.* **2021**, *6*, 38. https://doi.org/10.3390/tropicalmed6010038

Academic Editor: Natasha Rudenko

Received: 23 February 2021
Accepted: 12 March 2021
Published: 17 March 2021

1. Introduction

Lyme disease (LD) is a tick-borne disease (TBD) caused by bacteria from the *Borrelia burgdorferi sensu lato* group that can cause arthritic, dermatitis, or neurological manifestations [1–4]. Other common TBDs also include Babesiosis, Ehrlichiosis, Anaplasmosis, Encephalitis, and more [5–8]. Currently, TBDs are present in over 80 countries and may affect 35% of the world's population by 2050 [9]. In the meantime, the number of ticks that carry pathogens and can cause TBDs are ever-increasing [10–13]. Over the years, the reported TBD cases have spiked in various countries around the world [14–16]. Healthcare authorities like the Centers for Disease Control and Prevention (CDC) in the USA recognize that the real frequency of TBD cases in humans is much higher than the reported cases [17]. In 2018, the European Commission made headway by adding Lyme Neuroborreliosis to the list of diseases under the European Union's epidemiological surveillance [18]. Additionally, the European Parliament resolution recognized that the current TBD diagnostic tools are inaccurate, as they test for only one microbe at a time [19].

Globally, the CDC two-tier testing algorithm for LD stands undisputed by regulatory and healthcare authorities [20]. The literature is rife with evidence concerning the effectiveness of the CDC two-tier system for diagnosing LD [1,21,22]. The CDC recently revised its

LD testing algorithm by endorsing the use of two enzyme-linked immunosorbent assays (ELISAs) in both tiers [23]. However, the testing recommendations for other TBDs in LD patients is not clear, despite the growing evidence of coinfections in such patients [24]. An estimated 85% of LD patients can produce an immune response to TBD-related coinfections or opportunistic microbes [25]. Yet, 83% of all commercial TBD tests—for example, in the USA—are solely prescribed for LD [26]. The most putative diagnostic test manufacturers have popularized the use of a single test for a single disease following the Germ Theory [21,22,27]. As a result, the role, relevance, and requirements for a multiplex and multifunctional tool in the diagnosis of a complex disease like TBD are unclear for routine use in clinical laboratories.

Internationally, the research community has confirmed the likelihood of immune dysfunction in LD patients due to pathogenesis by Borrelia [28–33]. A TBD patient may experience an increase in disease severity, as Borrelia can sabotage, undermine, or trick the host immune system by evasion [32–34]. For example, Borrelia can repress the antigen-induced proliferation of lymphocyte cells or anti-Borrelia antibody response in immunocompromised patients [28,33]. Additionally, Borrelia can meddle with the kinetics and quality of B-cell and T-cell responses [34,35]. Hence, LD patients can present seronegative, delayed, or persistent antibody responses to Borrelia, indicating the complex nature of TBDs and a possible reason for misdiagnosed or undiagnosed cases [35–38]. Additionally, the regular discovery of novel and emerging TBD pathogens such as *Rickettsia monacensis*, Powassan virus, Omsk hemorrhagic virus, and others further complicates treatment for TBD patients without a holistic diagnostic tool [39].

A holistic diagnostic test may also help realize the need to institute a differential diagnosis in TBD testing recommendations. Patients with common symptoms like fever, headache, cough, and chills in the absence of laboratory evidence for LD could be misdiagnosed or remain undiagnosed for other conditions [40–42]. The prevalence of well-known TBD-related coinfections and opportunistic microbes are evident in individuals suffering from myalgia, fatigue, arthritis, and more [43]. For example, infection with *Bartonella* species can cause patients to complain about myalgia and fatigue [44]. Similarly, patients with fibromyalgia and chronic fatigue syndrome demonstrate an immune response to *Mycoplasma pneumoniae* or *Mycoplasma fermentans [45,46]*. While TBD is complicated to diagnose according to the literature mentioned above, will the use of comprehensive diagnostic tests prove practical to help reduce unrecognized patient cases? The goal of this study was to assess the need for a multiplex and multifunctional TBD immunoassay in routine clinical laboratory samples from Lyme disease and febrile patients a with (suspected) history of a tick bite.

2. Materials and Methods

2.1. Index Test and Interpretation

TICKPLEX® PLUS (herein, TICKPLEX®) is an ELISA index test used in this study that is a CE-IVD registered product (i.e., European In-Vitro Diagnostic Devices Directive (98/79/EC) compliant) manufactured in an ISO 13485:2016 accredited facility at Tezted Ltd, Jyväskylä, Finland. TICKPLEX® can measure the immunoglobulin M (IgM) and immunoglobulin G (IgG) immune responses in human serum samples against *Borrelia burgdorferi sensu lato* species in spirochete and persistent forms, coinfections, and opportunistic microbes. Mainly, TICKPLEX® includes *Borrelia burgdorferi sensu stricto*, Borrelia afzelii, and *Borrelia garinii* in spirochete and persistent form, *Babesia microti*, *Bartonella henselae*, *Ehrlichia chaffeensis*, *Rickettsia akari*, Coxsackievirus, Epstein–Barr virus, Human parvovirus B19, *Mycoplasma fermentans*, and Mycoplasma pneumoniae [25]. The clinical relevance for all TICKPLEX® microbes in TBD patients has been previously demonstrated [25]. The 150 human serums were tested blindly with the index test at Tezted Ltd. Normalized optical density values at 450 nm lower than 0.90, between 0.91 to 0.99, and higher than 1.00 were negative, borderline, and positive immune responses for all microbes, respectively.

2.2. Ethics Statement

United Medix Laboratories (Finland) provided anonymized and leftover human sera samples for research purposes. Sera sample included reference test results for LD, age, and gender for all patients. Following the General Data Protection Regulation (GDPR) [47], researchers at Tezted Ltd. did not have access to any private information (i.e., name, profession, or ethnicity) from the specimens that could be linked back to the patients. Hence, following the Declaration of Helsinki embodied in Common Rule set forth by the Code of Federal Regulations, USA, informed consent was not collected, as the present study was not considered as human subject research [48,49]. In Finland, the medical research act (488/1999) and the law on the medical usage of human organs, tissues, and cells (2.2.2001/101; section 20 (30.11.2012/689)) supports the use of leftover and deidentified human serum samples with consent from the collection unit [50,51]. United Medix Laboratories (Finland) was the collection unit for this study that contributed the deidentified human serum specimens according to their International Organization for Standardization (ISO) 15189 section 5.9.1. quality management system [52].

2.3. Reference Tests and Interpretation

Healthcare providers in Finland follow the CDC two-tier guidelines for LD diagnosis. Thus, Diasorin LIAISON® Borrelia chemiluminescence immunoassay (CLIA), Immunetics® C6 Lyme ELISA™ (C6 ELISA), and Mikrogen Diagnostik *recom*Bead Borrelia IgG 2.0 (IgG Blot) were used to confirm LD in human specimens. The CLIA test separately measures human IgM and IgG immune responses to *Borrelia burgdorferi sensu lato.* In contrast, the C6 ELISA measures human IgM and IgG combined immune reactions to the C6 synthetic peptide derived from the VlsE protein conserved in *Borrelia burgdorferi sensu stricto* or *Borrelia afzelii* and *Borrelia garinii*. For LD confirmation purposes, IgG Blot measured the human IgG immune response against *Borrelia burgdorferi sensu stricto*, *B. garinii*, *B. afzelii*, *B. bavariensis*, and *B. spielmanii*.

For the CLIA IgM test, arbitrary units per milliliter (AU/ml) less than 18, between 18 to 22, and more than 22 were considered negative, borderline, and positive immune responses, respectively. Similarly, for the CLIA IgG test, AU/ml less than 10, between 10 to 15, and more than 15 were considered negative, borderline, and positive immune responses, respectively. Like AU/ml, the C6 ELISA test utilized the Lyme Index (LI) with a normalized optical density value at 450 nm and a reference wavelength at 650 nm. As a result, LI less than 0.9, between 0.91 to 1.09, and more than 1.10 were considered negative, borderline, and positive immune responses, respectively. In the case of the IgG Blot test, normalized fluorescence intensities below 0.67, between 0.67 to 1.00, and above 1.00 were considered negative, borderline, and positive immune responses, respectively.

2.4. Patient Categorization

According to the CDC two-tier algorithm [24] for LD diagnosis and related test interpretation criteria, as mentioned above, the 150 human serum samples were organized in three different categories. LD-positive category (n = 48) included specimens with positive IgM or IgG immune responses to one (n = 7), two (n = 17), three (n = 9), or all four (n = 15) diagnostic tests. Category two included LD-negative (n = 30) serum samples with a negative immune response to all four tests (n = 15) and a positive immune response limited to the CLIA IgM or IgG test (n = 15). The last category included serum samples from patients with fever and a known or suspected history of a tick bite, i.e., from whom borrelia serology was requested (later, the febrile patient group) (n = 72). For the febrile patient group, the test results from the C6 ELISA and IgG Blot tests were not available.

2.5. Index Test and Interpretation

TICKPLEX® PLUS (herein, TICKPLEX®) is an ELISA index test used in this study that is a CE-IVD registered product manufactured in an ISO 13485:2016 accredited facility at Tezted Ltd. TICKPLEX® can measure IgM and IgG immune responses in human serum

samples against *Borrelia burgdorferi sensu lato* species in spirochete and persistent forms, coinfections, and opportunistic microbes. Mainly, TICKPLEX® includes *Borrelia burgdorferi sensu stricto*, *Borrelia afzelii*, and *Borrelia garinii* in spirochete and persistent form, *Babesia microti*, *Bartonella henselae*, *Ehrlichia chaffeensis*, *Rickettsia akari*, Coxsackievirus, Epstein–Barr virus, Human parvovirus B19, *Mycoplasma fermentans*, and Mycoplasma pneumoniae [25]. The clinical relevance for all TICKPLEX® microbes in TBD patients has been previously demonstrated [25]. The 150 human serums were tested blindly with the index test at Tezted Ltd. Normalized optical density values at 450 nm lower than 0.90, between 0.91 to 0.99, and higher than 1.00 were negative, borderline, and positive immune responses for all microbes, respectively.

2.6. Statistical Analysis

For quality control purposes, an inter-plate and inter-operator precision analysis was conducted by assessing the coefficient of variance [53] (CV %) on the optical density values for IgM/IgG plate controls and all microbial antigens on TICKPLEX®. To assess the CV % for index test microbial antigens, the negative serum control (TEZ1) in the kit was repeatedly performed in each plate by each operator. Equations (1)–(3) were utilized to calculate the proportion of positive (PA), negative (NA), and overall (OA) agreement, respectively, among the reference tests and between the reference tests versus (vs.) index test [54]. The PA, NA, and OA agreements among the reference tests and between reference tests with the index test were combined for the IgM and IgG immune responses. In Equations (1)–(3), the letters a, b, c, and d stand for true positives, false positives, false negatives, and true negatives, respectively. Further, the reliability for each PA and NA comparison was evaluated by calculating Cohen's kappa (k) with a 95% confidence interval [54,55].

$$PA = \frac{2a}{2a + b + c} \quad (1)$$

$$NA = \frac{2d}{2d + b + c} \quad (2)$$

$$OA = \frac{a + d}{a + b + c + d} \quad (3)$$

Cohen's k ranges from -1 to $+1$, wherein k values ≤ 0 indicates no agreement, 0.01–0.20 as none to a slight agreement, 0.21–0.40 as fair agreement, 0.41–0.60 as moderate agreement, 0.61–0.80 as substantial agreement, and 0.81–1.00 as almost perfect agreement [55]. Proportionate positive and negative agreements, along with Cohen's k, were calculated using the EPITOOLS diagnostic test evaluation and comparison calculator. The inter-rater reliability and proportional agreement analysis between various tests were carried out using just LD-positive and -negative patient groups. Further, Fisher's exact test was used to assess the statistical differences in IgM or IgG immune responses between the LD (positive and negative) and febrile patient groups. The two-tailed p-values for the Fisher's exact test were calculated using GraphPad (https://www.graphpad.com/quickcalcs/contingency1/ (accessed on 28 May 2019). Fisher's exact test results with p-values < 0.05 were considered statistically associated or dependent [56].

3. Results

The United Medix Laboratories in Finland collected specimens from LD-positive (n = 48) and LD-negative (n = 30) patients and from febrile patients from whom borrelia serology was requested (n = 72). The samples were collected amid routine clinical diagnostic services (convenience sampling) in the summer of 2018, beginning from late-May to mid-September. On average, patients were 42, 39, and 36 years old in the LD-positive, LD-negative, and febrile groups, respectively. The LD-positive patient group included 27 male and 21 female human serum samples. Likewise, the LD-negative group included specimens from 15 male and 15 female patients. Lastly, specimens from the febrile patients consisted of 31 male and 41 female human specimens. Overall, the average age for 73 male and

77 female serum samples was 39 years. Further, the inter-plate and inter-operator CV % for IgM and IgG on the index test were 6.280% and 4.692%, respectively. Additionally, the CV % for the internal negative control (TEZ1) was observed to be ≤15% for all microbial antigens on the index test.

Figure 1 illustrates the PA, NA, OA, and Cohen's *k* among the reference tests and between the reference tests with the index test. The PA for the individual reference or index test ranged between 53% for IgG Blot vs. TICKPLEX® to 72% for CLIA IgM/IgG vs. C6 ELISA. Similarly, the lowest NA was observed for CLIA IgM/IgG vs. TICKPLEX® (49%) and the highest between IgG Blot vs. TICLPLEX (76%). The OA ranged from 55% for CLIA IgM/IgG vs. TICKPLEX® to 73% between C6 ELISA vs. IgG Blot. Except for a moderate Cohen's *k* agreement between C6 ELISA vs. IgG Blot (*k* = 0.45), all the other individual test combinations displayed fair Cohen's *k* agreements (*k* = 0.12 to 0.31). Among the different test comparisons individually, the average PA, NA, and OA were 63.5%, 62.33%, and 64%, respectively. As mentioned earlier, four Lyme disease tests (i.e., reference tests) were used to confirm Borrelia infection according to the CDC two-tier criteria. A substantial Cohen's *k* agreement was observed between the commercial two-tiered tests vs. TICKPLEX® (*k* = 0.74). The PA, NA, and OA for comparisons between all reference tests and TICKPLEX® were 86%, 88%, and 87%, respectively (Figure 1).

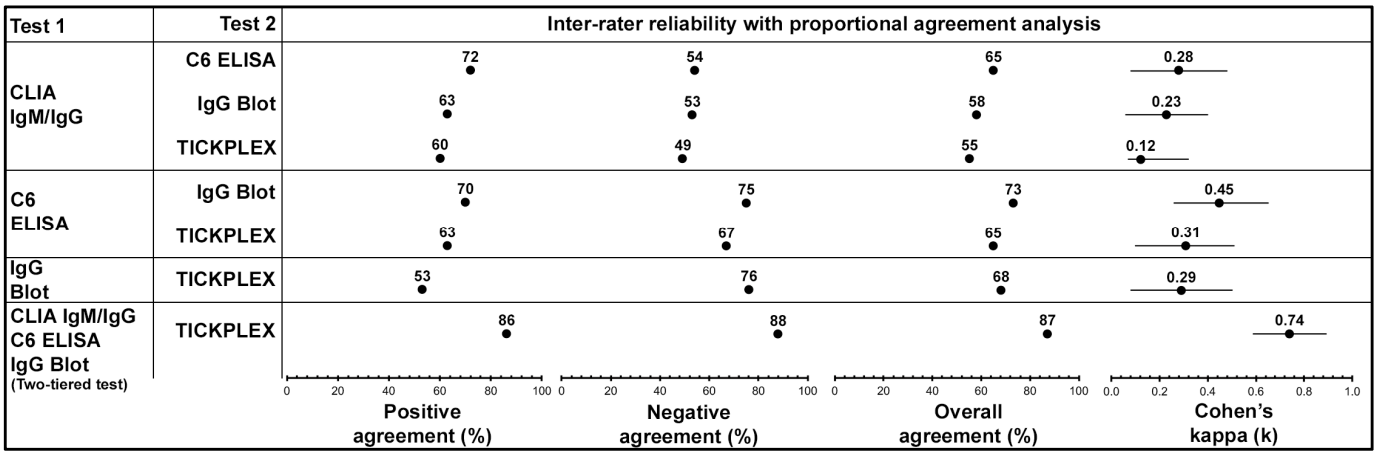


Figure 1. TICKPLEX® can aid replace the need for four Lyme disease diagnostic tests, as the index test clinical performance substantially agrees with the Centers for Disease Control and Prevention (CDC) two-tier system. The collective immunoglobulin M/immunoglobulin G (IgM/IgG) inter-rater reliability (i.e., Cohen's *k*) and proportional agreement analysis (i.e., positive, negative, and overall agreement) among reference tests and between the reference tests with the index test. Herein, reference tests refer to Diasorin LIAISON®Borrelia chemiluminescence immunoassay (CLIA), Immunetics®C6 Lyme ELISATM (C6 ELISA), and Mikrogen Diagnostik *recom*Bead Borrelia IgG 2.0 (IgG Blot). Similarly, the index test refers to TICKPLEX® PLUS (TICKPLEX®). Further, Cohen's *k* ranges from −1 to +1, wherein *k* values ≤ 0 indicates no agreement, 0.01–0.20 as none to a slight agreement, 0.21–0.40 as fair agreement, 0.41–0.60 as moderate agreement, 0.61–0.80 as substantial agreement, and 0.81–1.00 as almost perfect agreement. The present figure uses the reference and index test results from Lyme disease-positive (*n* = 48) and -negative (*n* = 30) groups.

In addition to Lyme disease, the LD-positive, LD-negative, and febrile patient groups were also tested against TBD related coinfections and opportunistic microbes using TICKPLEX®. Figure 2 is a cooccurrence heat map indicating the percentage of IgM or IgG immune responses by LD (positive and negative) and febrile patient groups to TICKPLEX® antigens. Borrelia spirochete species and persistent forms witnessed the most significant percentage of IgM and IgG immune responses in both patient groups. Apart from Borrelia, an average 2% for IgM and 8% for IgG immune responses were noted by LD specimens to coinfections and opportunistic microbes related to TBD (herein other microbes). Likewise, on average, 4% for IgM and 6% for IgG immune responses were observed for febrile patient samples against other TBD-related microbes. Overall, a statistical association or dependence was observed

between LD and the febrile patient group's IgM and IgG responses to the Epstein–Barr virus and Borrelia spirochete species, respectively (Figure S1). No association in IgM or IgG immune responses with the remaining TICKPLEX® antigens were noted between the LD and febrile patient groups (Figure S1).

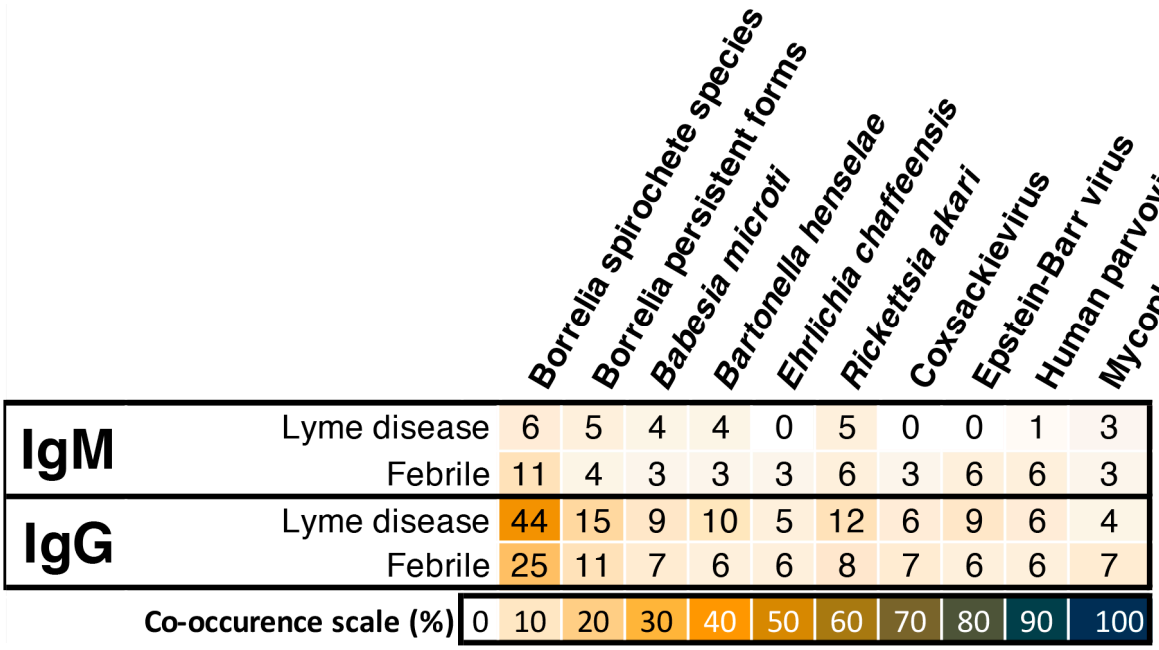


Figure 2. Screening of febrile patients for tick-borne diseases could be a missed opportunity at reducing misdiagnosed and undiagnosed patient cases, as their positive IgM and IgG immune response percentages are similar to the Lyme disease group. Borrelia spirochete species and Borrelia persistent forms refer to *Borrelia burgdorferi sensu stricto*, *Borrelia afzelii*, and *Borrelia garinii* in spirochete and persistent forms, respectively. Similarly, Mycoplasma species refers to *Mycoplasma fermentans* and *Mycoplasma pneumoniae.*

The IgM and IgG immune responses by the LD and febrile patient groups to Borrelia alone, Borrelia and other microbes, and just other microbes in the index test were further analyzed (Figure 3). For IgM, 1%, 6%, and 0% LD patients responded to only Borrelia, Borrelia and other microbes, and only other microbes, respectively (Figure 3A). Similarly, 4%, 7%, and 4% of the febrile patient specimens produced an IgM response against only Borrelia, Borrelia and other microbes, and only other microbes, respectively (Figure 3A). In the case of IgG immune responses by LD patients, 28%, 15%, and 4% of the patients responded to only Borrelia, Borrelia and other microbes, and only other microbes, respectively (Figure 3B). Likewise, 14%, 11%, and 0% of the febrile patient specimens produced IgG response against only Borrelia, Borrelia and other microbes, and only other microbes, respectively (Figure 3B). A statistical association was observed between the LD and febrile patient groups' IgG responses to only Borrelia (Figure 3).

Figure S2 demonstrates the percentage of LD or febrile patient IgM and IgG immune responses to the number of other microbes along with Borrelia. The IgM or IgG immune responses to Borrelia and one other microbe was the most significant percentage of the reaction seen in both the LD and febrile patient groups. In the case of the LD patient group, 3% for IgM and 6% for IgG responded to Borrelia and one other microbe, respectively. Similarly, 4% and 3% of the febrile patients produced IgM and IgG responses to Borrelia and one other microbe, respectively. Not more than 1% of the LD or febrile patient specimens in IgM or IgG responded to Borrelia and two other microbes to seven other microbes.

Remarkably, the second most significant percentage of IgM or IgG immune responses was seen in both the LD and febrile patient specimens for Borrelia and eight other microbes. Approximately 4% IgM or IgG immune responses were noted from the LD and febrile

patient groups against Borrelia and eight other microbes. An IgM or IgG immune response to Borrelia and eight other microbes primarily responded to all ten TICKPLEX® antigens. At random, a serum sample with IgM and IgG immune response to Borrelia and eight other microbes was selected and serially diluted on TICKPLEX®. As a result, a clear dose-dependent response was observed (Figure S3).

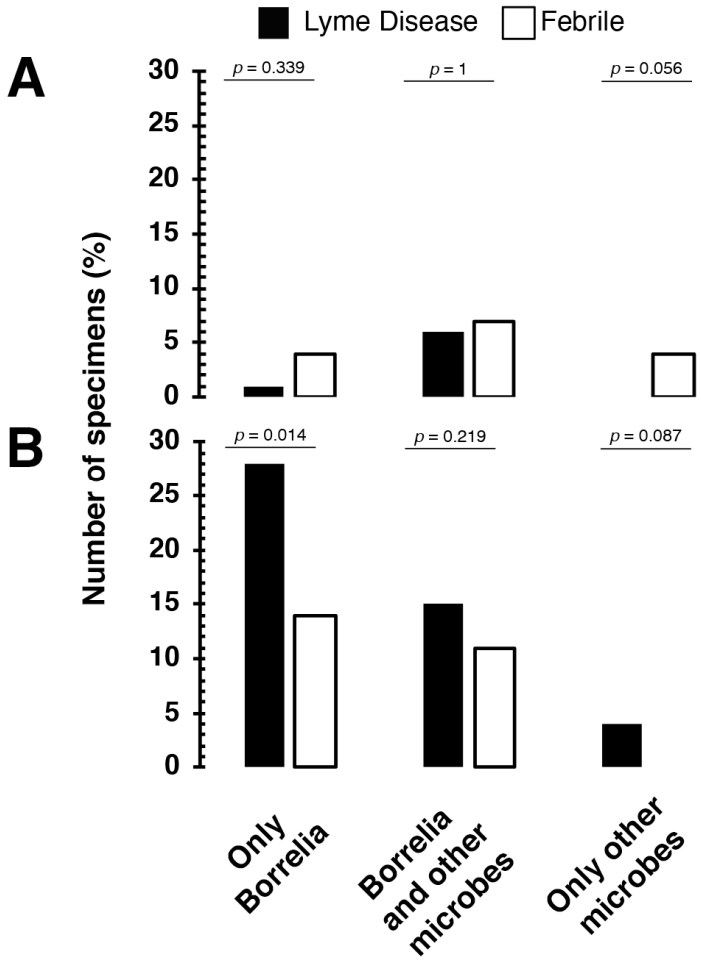


Figure 3. Lyme disease and febrile patients produce (**A**) IgM and (**B**) IgG immune responses to Borrelia and multiple coinfections and opportunistic microbes related to tick-borne diseases. Other microbes refer to *Babesia microti*, *Bartonella henselae*, *Ehrlichia chaffeensis*, *Rickettsia akari*, Coxsackievirus, Epstein–Barr virus, Human parvovirus B19, *Mycoplasma fermentans*, and *Mycoplasma pneumoniae* in the index test. The *p*-value originates from the Fisher's exact test that was used to assess the statistical differences in IgM or IgG immune responses between Lyme disease (LD) (positive and negative) and febrile patient groups. The two-tailed *p*-values for the Fisher's exact test were calculated using GraphPad (https://www.graphpad.com/quickcalcs/contingency1/ (accessed on 28 May 2019)). The Fisher's exact test results with *p*-values < 0.05 were considered statistically associated or dependent.

4. Discussion

To evaluate the use for a multiplex and multifunctional TBD immunoassay in a routine clinical laboratory, LD-positive (n = 48), LD-negative (n = 30), and febrile (n = 72) patient specimens were tested against TICKPLEX® microbial antigens for their IgM and IgG immune responses. The clinical performance of TICKPLEX® (index test) for testing LD was compared to four reference tests (CLIA IgM and IgG, C6 ELISA, and IgG Blot) used at the United Medix Laboratories in Finland following the CDC two-tier criteria. Individual comparisons among the reference tests and between the reference with the index tests resulted in an average PA, NA, and OA of 63.5%, 62.33%, and 64%, respectively

(Figure 1). A substantial Cohen's *k* agreement (*k* = 0.74) was mainly observed when the clinical outcome from all four reference tests was compared with the TICKPLEX® results (Figure 1). A comparison between the commercial CDC two-tiered LD testing system with TICKPLEX® produced 86% PA, 88% NA, and 87% OA (Figure 1).

Variations in the PA, NA, or OA among the LD diagnostic tests is a rule rather than the exception, because several in vitro diagnostic test manufacturers utilize different Borrelia proteins [21,22,27,57]. For example, the positivity rate for LD patients with an Erythema Migrans rash can range from 18% to 53% for whole-cell antigen LD tests vs. 31% to 50% for recombinant antigen LD tests [57]. Generally, diagnostic test sensitivities improve from the early to late LD stages [21,22,57]. In later LD stages like neuroborreliosis, the positivity rate can vary from 41% to 86% for whole-cell antigen LD tests and 49% to 81% for recombinant antigen LD tests [57]. Additionally, with regards to the CDC two-tier testing system, a PA among commercial LD tests can vary from 5% to 98.5%, and a NA can range from 28.6% to 100% [21]. Overall, at any given LD stage, the average accuracy for LD diagnostic tests is 62.3% [21,27]. Similar accuracy averages in this study were observed among the reference tests and between the reference and index tests (Figure 1).

While the accuracy averages for LD diagnostic tests between this study and the literature are comparable, the study findings herein also indicated that TICKPLEX® is a suitable replacement for the CLIA IgM/IgG, C6 ELISA, and IgG Blot reference tests. A dramatic increase in correlations between the commercial CDC two-tiered LD tests and TICKPLEX® is connected to a consistent PA (60% to 63%) with CLIA IgM/IgG and C6 ELISA plus a high NA (76%) with the IgG Blot test (Figure 1). The C6 ELISA demonstrates a similarly dramatic change in a PA and NA when compared with either an individual LD test or a CDC two-tiered testing system [21]. A previous comparison between the C6 ELISA and CLIA IgM/IgG tests yielded 70% PA and 99.1% NA [21]. However, the current study demonstrated 72% PA and only 54% NA between the C6 ELISA and CLIA IgM/IgG tests (Figure 1). Nevertheless, a 98.5% PA and 49% NA was evident between the C6 ELISA and CDC two-tiered tests, which included the Wampole Bb (IgG/IgM) ELISA test system, MarDx Lyme Disease (IgG and IgM), and Marblot strip test system [21]. As a result, the PA, NA, and OA of LD tests strongly depend on the type of reference test used for comparison [21,22,27,57].

The TICKPLEX® results also indicated that 6% to 15% of the LD individuals responded to TBD-related coinfections and opportunistic microbes (Figure 2). Traditionally, a TBD-linked opportunistic infection in a LD patient could be the result of a vulnerable immune system due to a prolonged TBD infection [32,33,58]. Immune responses by LD patients to multiple other TBD microbes with or without Borrelia demonstrate that TBD is not limited to just LD in Finland (Figures 2 and 3). In several other countries, like Germany, Sweden, the Netherlands, and more, 4% to 60% of LD patients can suffer from LD and TBD-related coinfections [59–61]. Multiple TBD-associated infections in LD patients primarily originate from ticks that can carry over 120 distinct bacterial and other microbial species [62]. In various regions of Finland, the cooccurrence percentage for multiple pathogens in ticks ranges from 1.02% to 28.3% [11–13,63,64]. In 2004, a Finnish LD patient suffered from fatal Babesiosis [65]. Therein, no research articles on PubMed elucidated the relevance of TBD-related coinfections or opportunistic microbes in Finland.

Furthermore, the IgG immune responses were statistically correlated between the LD and febrile patient groups (Figure S1 and Figure 3). Moreover, 7% to 11% of the febrile patients reacted to other TBD-related microbes (Figures 2 and 3). The current study demonstrated that individuals with fever and a putative history of a tick bite can respond to TBD microbes similar to LD patients (Figures 2 and 3). A misdiagnosis of early LD as summer flu is an understudied topic in the field of TBDs [41]. Not all LD patients demonstrate an Erythema Migrans (EM) rash or produce detectable antibodies in the first two to four weeks. A misdiagnosis is probable for nearly 16% of LD cases that do not display an EM rash [66]. Additionally, 60% of early-stage LD individuals receive a negative LD diagnostic test result, as they do not develop a detectable level of antibodies and are

therefore susceptible to misdiagnosis [67]. A TBD infection can cause nonspecific febrile illness wherein individuals may suffer from LD (11%), human granulocytic ehrlichiosis (13%), or coinfections (3%) [68].

Lastly, an IgM or IgG immune response to all TICKPLEX® antigens by 4% of the LD and febrile patient groups is an unexpected finding in this study (Figure S2). The unspecific binding of human specimens to recombinant proteins or blocking agents on an ELISA test is a plausible interpretation [69,70]. However, all TICKPLEX® antigens comprise of either whole-cell lysates or synthetic peptides and not recombinant proteins. Secondly, a sera sample from 4% of the LD and febrile patients at random was serially diluted to correlate the declining antibody concentration with optical density values. In the presence of an unspecific reaction, a serial dilution of sera specimen will not make any difference on the resulting optical density value. Figure S3 indicates no unspecific binding on TICKPLEX® for IgM and IgG. Immune evasion and host immune response suppression, modulation, or subversion by Borrelia in LD patients is a common finding [28,30,32,33,71–73]. For example, Borrelia can trick the host immune system into producing a strong yet inadequate response while it evades the lymph nodes [34]. We postulate that a universally positive IgM or IgG immune response in TBD patients could be the result of a B-cell-related immune dysfunction, such as unspecific B-cell activation [29,34,74].

A noticeable improvement to the current study would be to increase the overall sample size and improve the statistical confidence in the findings. In the future, the study design could also include a comparison between TICKPLEX® non-Borrelia antigens and related reference tests in a routine lab clinical setting. Additionally, a multicenter prospective study approach with several TBD disease patient groups would aid in a health economic assessment and awareness for TBD diagnosis with TICKPLEX®. Furthermore, a systematic investigation is required to assess the significance and prevalence of TBD patients with an IgM or IgG-positive immune response to every microbial protein (universally positive).

In conclusion, the present study makes evident that the clinical performance of Borrelia spirochete species and Borrelia persistent forms on TICKPLEX® is in-line with the industry standard PA, NA, and OA. Additionally, the unique Borrelia protein combination in TICKPLEX® can reduce the need from four tests for a LD diagnosis to just one test. Furthermore, in a routine clinical lab, a multiplex and multifunctional test can help detect TBD-related coinfections and opportunistic microbes in LD patients. Moreover, the screening of febrile or summer flu patients for TBDs could be a missed opportunity at reducing misdiagnosed and undiagnosed patient cases.

Supplementary Materials: The following are available online at https://www.mdpi.com/2414-6366/6/1/38/s1, Figure S1. Statistical association or dependence was observed between Lyme disease and febrile patient groups' IgM and IgG responses to the Epstein–Barr virus and Borrelia spirochete species, respectively. Borrelia spirochete species and Borrelia persistent forms refer to *Borrelia burgdorferi sensu stricto*, *Borrelia afzelii*, and *Borrelia garinii* in spirochete and persistent forms, respectively. Similarly, Mycoplasma species refers to *Mycoplasma fermentans* and *Mycoplasma pneumoniae.* The *p*-value originates from the Fisher's exact test that was used to assess the statistical differences in the IgM or IgG immune responses between the LD (positive and negative) and febrile patient groups. The two-tailed *p* values for the Fisher's exact test was calculated using GraphPad (https://www.graphpad.com/quickcalcs/contingency1/ (accessed on 28 May 2019)). Fisher's exact test results with p-values < 0.05 were considered statistically associated or dependent. Figure S2. Lyme disease and febrile patient specimens demonstrated (A) IgM and (B) IgG immune responses for up to eight other microbes with Borrelia using the TICKPLEX® test. In the present figure, the other microbes refer to *Babesia microti*, *Bartonella henselae*, *Ehrlichia chaffeensis*, *Rickettsia akari*, Coxsackievirus, Epstein–Barr virus, Human parvovirus B19, *Mycoplasma fermentans*, and *Mycoplasma pneumoniae* in the index test. Figure S3. No (A) IgM or (B) IgG unspecific binding is observed in the TICKPLEX® test. Borrelia spirochete species and Borrelia persistent forms refer to *Borrelia burgdorferi sensu stricto*, *Borrelia afzelii*, and *Borrelia garinii* in spirochete and persistent forms, respectively. Similarly, Mycoplasma species refers to *Mycoplasma fermentans* and *Mycoplasma pneumoniae*. Table S1. Normalized IgM optical density values for Lyme disease-positive (sera ID 1-48), -negative (sera ID

49-78), and febrile patients (sera ID 79-150) from the index test. Table S2. Normalized IgG optical density values for Lyme disease-positive (sera ID 1-48), -negative (sera ID 49-78), and febrile patients (sera ID 79-150) from the index test.

Author Contributions: Conceptualization, K.G., T.S.J., and L.G.; data curation, K.G., S.F., and L.G.; formal analysis, K.G., S.F., and L.G.; funding acquisition, L.G.; investigation, K.G. and L.G.; methodology, K.G., T.S.J., and L.G.; project administration, T.S.J. and L.G.; resources, T.S.J., S.F., and L.G.; supervision, T.S.J. and L.G.; validation, K.G., T.S.J., S.F., and L.G.; visualization, K.G. and L.G.; writing—original draft, K.G. and L.G.; and writing—review and editing, K.G., T.S.J., S.F., and L.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Schwartz Foundation, which is a philanthropic organization in the USA, supported the present study. The foundation had no role in the study design, data collection, analyses, the decision to publish, or preparation of the manuscript.

Institutional Review Board Statement: Ethical review and approval were waived for this study in accordance with the ethics statement presented in the Materials and Methods section.

Informed Consent Statement: Patient consent was waived in accordance with the ethics statement presented in the Materials and Methods section.

Data Availability Statement: Data was contained within the article or Supplementary Materials. All normalized optical density values resulting from the index test were provided in the Supplementary Materials, Tables S1 and S2.

Conflicts of Interest: K.G and L.G. have a financial and business interest in Tezted Ltd. Further, S.J and S.F do not have commercial or financial relationships that could be construed as potential conflicts of interest. The funding source mentioned in the Funding and Acknowledgements section and Tezted Ltd. had no role in the experimental design, reporting of the results, or the decision to publish.

References

1. Aguero-Rosenfeld, M.E.; Wang, G.; Schwartz, I.; Wormser, G.P. Diagnosis of lyme borreliosis. *Clin. Microbiol Rev.* **2005**, *18*, 484–509. [CrossRef]
2. Wilske, B. Epidemiology and diagnosis of Lyme borreliosis. *Ann. Med.* **2005**, *37*, 568–579. [CrossRef]
3. Aberer, E.; Kersten, A.; Klade, H.; Poitschek, C.; Jurecka, W. Heterogeneity of Borrelia burgdorferi in the skin. *Am. J. Dermatopathol.* **1996**, *18*, 571–579. [CrossRef] [PubMed]
4. Halperin, J.J. Lyme Disease: Neurology, Neurobiology, and Behavior. *Clin. Infect. Dis.* **2014**, *58*, 1267–1272. [CrossRef]
5. Lempereur, L.; Shiels, B.; Heyman, P.; Moreau, E.; Saegerman, C.; Losson, B.; Malandrin, L. A retrospective serological survey on human babesiosis in Belgium. *Clin. Microbiol. Infect.* **2015**, *21*, 96.e1–96.e7. [CrossRef] [PubMed]
6. Grab, D.J.; Nyarko, E.; Barat, N.C.; Nikolskaia, O.V.; Dumler, J.S. Anaplasma phagocytophilum-Borrelia burgdorferi Coinfection Enhances Chemokine, Cytokine, and Matrix Metalloprotease Expression by Human Brain Microvascular Endothelial Cells. *Clin. Vaccine Immunol.* **2007**, *14*, 1420–1424. [CrossRef] [PubMed]
7. Ismail, N.; Bloch, K.C.; McBride, J.W. Human Ehrlichiosis and Anaplasmosis. *Clin. Lab. Med.* **2010**, *30*, 261–292. [CrossRef] [PubMed]
8. Steffen, R. Epidemiology of tick-borne encephalitis (TBE) in international travellers to Western/Central Europe and conclusions on vaccination recommendations. *J. Travel Med.* **2016**, *23*. [CrossRef]
9. Davidsson, M. The Financial Implications of a Well-Hidden and Ignored Chronic Lyme Disease Pandemic. *Healthcare* **2018**, *6*, 16. [CrossRef]
10. Karim, S.; Budachetri, K.; Mukherjee, N.; Williams, J.; Kausar, A.; Hassan, M.; Adamson, S.; Dowd, S.E.; Apanskevich, D.; Arijo, A.; et al. A study of ticks and tick-borne livestock pathogens in Pakistan. *PLoS Neglect. Trop. D* **2017**, *11*, e0005681. [CrossRef]
11. Laaksonen, M.; Klemola, T.; Feuth, E.; Sormunen, J.J.; Puisto, A.; Mäkelä, S.; Penttinen, R.; Ruohomäki, K.; Hänninen, J.; Sääksjärvi, I.E.; et al. Tick-borne pathogens in Finland: Comparison of Ixodes ricinus and I. persulcatus in sympatric and parapatric areas. *Parasite Vector* **2018**, *11*, 556. [CrossRef] [PubMed]
12. Sormunen, J.J.; Penttinen, R.; Klemola, T.; Hänninen, J.; Vuorinen, I.; Laaksonen, M.; Sääksjärvi, I.E.; Ruohomäki, K.; Vesterinen, E.J. Tick-borne bacterial pathogens in southwestern Finland. *Parasite Vector* **2016**, *9*, 168. [CrossRef] [PubMed]
13. Sormunen, J.J.; Andersson, T.; Aspi, J.; Bäck, J.; Cederberg, T.; Haavisto, N.; Halonen, H.; Hänninen, J.; Inkinen, J.; Kulha, N.; et al. Monitoring of ticks and tick-borne pathogens through a nationwide research station network in Finland. *Ticks Tick Borne Dis.* **2020**, *11*, 101449. [CrossRef]
14. DeLong, A.; Hsu, M.; Kotsoris, H. Estimation of cumulative number of post-treatment Lyme disease cases in the US, 2016 and 2020. *BMC Public Health* **2019**, *19*, 352. [CrossRef] [PubMed]
15. Kugeler, K.J.; Eisen, R.J. Challenges in Predicting Lyme Disease Risk. *JAMA Netw. Open* **2020**, *3*, e200328. [CrossRef]

16. Stone, B.L.; Tourand, Y.; Brissette, C.A. Brave New Worlds: The Expanding Universe of Lyme Disease. *Vector-Borne Zoonot* **2017**, *17*, 619–629. [CrossRef]
17. Centers for Disease Control and Prevention (CDC). How Many People Get Lyme Disease? Available online: https://www.cdc.gov/lyme/stats/humancases.html (accessed on 16 February 2019).
18. European Centre for Disease Prevention and Control. ECDC Comment: European Commission Updates Communicable Disease Surveillance List—Lyme Neuroborreliosis Now under EU/EEA Surveillance. Available online: https://www.ecdc.europa.eu/en/news-events/ecdc-comment-european-commission-updates-communicable-disease-surveillance-list-lyme (accessed on 28 May 2019).
19. Parliament, E. European Parliament Resolution of 15 November 2018 on Borrelia Infection. Strasbourg 2018. Available online: https://www.europarl.europa.eu/doceo/document/TA-8-2018-0465_SV.html?redirect (accessed on 28 May 2019).
20. Eldin, C.; Raffetin, A.; Bouiller, K.; Hansmann, Y.; Roblot, F.; Raoult, D.; Parola, P. Review of European and American guidelines for the diagnosis of Lyme borreliosis. *Médecine Mal. Infect* **2019**, *49*, 121–132. [CrossRef]
21. Waddell, L.A.; Greig, J.; Mascarenhas, M.; Harding, S.; Lindsay, R.; Ogden, N. The Accuracy of Diagnostic Tests for Lyme Disease in Humans, A Systematic Review and Meta-Analysis of North American Research. *PLoS ONE* **2016**, *11*, e0168613. [CrossRef]
22. Leeflang, M.M.; Ang, C.W.; Berkhout, J.; Bijlmer, H.A.; Bortel, W.V.; Brandenburg, A.H.; Burgel, N.D.V.; Dam, A.P.V.; Dessau, R.B.; Fingerle, V.; et al. The diagnostic accuracy of serological tests for Lyme borreliosis in Europe: A systematic review and meta-analysis. *BMC Infect Dis.* **2016**, *16*, 140. [CrossRef] [PubMed]
23. Branda, J.A.; Strle, K.; Nigrovic, L.E.; Lantos, P.M.; Lepore, T.J.; Damle, N.S.; Ferraro, M.J.; Steere, A.C. Evaluation of Modified 2-Tiered Serodiagnostic Testing Algorithms for Early Lyme Disease. *Clin. Infect. Dis.* **2017**, *64*, 1074–1080. [CrossRef] [PubMed]
24. Centers for Disease Control and Prevention (CDC). Recommendations for test performance and interpretation from the Second National Conference on Serologic Diagnosis of Lyme Disease. *MMWR. Morb. Mortal. Wkly. Rep.* **1995**, *44*, 590–591.
25. Garg, K.; Meriläinen, L.; Franz, O.; Pirttinen, H.; Quevedo-Diaz, M.; Croucher, S.; Gilbert, L. Evaluating polymicrobial immune responses in patients suffering from tick-borne diseases. *Sci. Rep.* **2018**, *8*, 15932. [CrossRef]
26. Connally, N.P.; Hinckley, A.F.; Feldman, K.A.; Kemperman, M.; Neitzel, D.; Wee, S.-B.; White, J.L.; Mead, P.S.; Meek, J.I. Testing practices and volume of non-Lyme tickborne diseases in the United States. *Ticks Tick-Borne Dis.* **2016**, *7*, 193–198. [CrossRef] [PubMed]
27. Cook, M.J.; Puri, B.K. Commercial test kits for detection of Lyme borreliosis: A meta-analysis of test accuracy. *Int. J. Gen. Med.* **2016**, *9*, 427–440. [CrossRef] [PubMed]
28. Chiao, J.W.; Pavia, C.; Riley, M.; Altmann-Lasekan, W.; Abolhassani, M.; Liegner, K.; Mittelman, A. Antigens of Lyme disease of spirochaete Borrelia burgdorferi inhibits antigen or mitogen-induced lymphocyte proliferation. *FEMS Immunol. Med. Mic.* **1994**, *8*, 151–155. [CrossRef]
29. Aberer, E.; Koszik, F.; Silberer, M. Why is chronic Lyme borreliosis chronic? *Clin. Infect. Dis.* **1997**, *25*, S64–S70. [CrossRef] [PubMed]
30. Chung, Y.; Zhang, N.; Wooten, R.M. Borrelia burgdorferi elicited-IL-10 suppresses the production of inflammatory mediators, phagocytosis, and expression of co-stimulatory receptors by murine macrophages and/or dendritic cells. *PLoS ONE* **2013**, *8*, e84980. [CrossRef] [PubMed]
31. Jarefors, S.; Janefjord, C.K.; Forsberg, P.; Jenmalm, M.C.; Ekerfelt, C. Decreased up-regulation of the interleukin-12Rbeta2-chain and interferon-gamma secretion and increased number of forkhead box P3-expressing cells in patients with a history of chronic Lyme borreliosis compared with asymptomatic Borrelia-exposed individuals. *Clin. Exp. Immunol.* **2007**, *147*, 18–27. [CrossRef] [PubMed]
32. Elsner, R.A.; Hastey, C.J.; Baumgarth, N. CD4+ T cells promote antibody production but not sustained affinity maturation during Borrelia burgdorferi infection. *Infect Immun.* **2015**, *83*, 48–56. [CrossRef]
33. Elsner, R.A.; Hastey, C.J.; Olsen, K.J.; Baumgarth, N. Suppression of Long-Lived Humoral Immunity Following Borrelia burgdorferi Infection. *PLoS Pathog.* **2015**, *11*, e1004976. [CrossRef]
34. Tunev, S.S.; Hastey, C.J.; Hodzic, E.; Feng, S.; Barthold, S.W.; Baumgarth, N. Lymphoadenopathy during lyme borreliosis is caused by spirochete migration-induced specific B cell activation. *PLoS Pathog.* **2011**, *7*, e1002066. [CrossRef] [PubMed]
35. Hastey, C.J.; Elsner, R.A.; Barthold, S.W.; Baumgarth, N. Delays and diversions mark the development of B cell responses to Borrelia burgdorferi infection. *J. Immunol.* **2012**, *188*, 5612–5622. [CrossRef] [PubMed]
36. Aberer, E.; Schwantzer, G. Course of Antibody Response in Lyme Borreliosis Patients before and after Therapy. *ISRN Immunol* **2012**, 1–4. [CrossRef]
37. Lomholt, H.; Lebech, A.M.; Hansen, K.; Brandrup, F.; Halkier-Sørensen, L. Long-term serological follow-up of patients treated for chronic cutaneous borreliosis or culture-positive erythema migrans. *Acta Derm. Venereol.* **2000**, *80*, 362–366.
38. Tracy, K.E.; Baumgarth, N. Borrelia burgdorferi Manipulates Innate and Adaptive Immunity to Establish Persistence in Rodent Reservoir Hosts. *Front Immunol.* **2017**, *8*, 116. [CrossRef]
39. Vayssier-Taussat, M.; Kazimirova, M.; Hubalek, Z.; Hornok, S.; Farkas, R.; Cosson, J.-F.; Bonnet, S.; Vourch, G.; Gasqui, P.; Mihalca, A.D.; et al. Emerging horizons for tick-borne pathogens: From the "one pathogen-one disease" vision to the pathobiome paradigm. *Future Microbiol.* **2015**, *10*, 2033–2043. [CrossRef] [PubMed]
40. Aucott, J.; Morrison, C.; Munoz, B.; Rowe, P.C.; Schwarzwalder, A.; West, S.K. Diagnostic challenges of early Lyme disease: Lessons from a community case series. *BMC Infect Dis.* **2009**, *9*, 79. [CrossRef]

41. Aucott, J.N.; Seifter, A. Misdiagnosis of early Lyme disease as the summer flu. *Orthop. Rev.* **2011**, *3*, e14. [CrossRef]
42. Johnson, L.; Wilcox, S.; Mankoff, J.; Stricker, R.B. Severity of chronic Lyme disease compared to other chronic conditions: A quality of life survey. *PeerJ* **2014**, *2*, e322. [CrossRef]
43. Berghoff, W. Chronic Lyme Disease and Co-infections: Differential Diagnosis. *Open Neurol. J.* **2012**, *6*, 158–178. [CrossRef]
44. Maggi, R.G.; Mozayeni, B.R.; Pultorak, E.L.; Hegarty, B.C.; Bradley, J.M.; Correa, M.; Breitschwerdt, E.B. Bartonella spp. bacteremia and rheumatic symptoms in patients from Lyme disease-endemic region. *Emerg. Infect Dis.* **2012**, *18*, 783–791. [CrossRef] [PubMed]
45. Nasralla, M.; Haier, J.; Nicolson, G. Multiple Mycoplasmal Infections Detected in Blood of Patients with Chronic Fatigue Syndrome and/or Fibromyalgia Syndrome. *Eur. J. Clin. Microbiol. Infect Dis.* **1999**, *18*, 859–865. [CrossRef]
46. Nicolson, G.L.; Gan, R.; Haier, J. Multiple co-infections (Mycoplasma, Chlamydia, human herpes virus-6) in blood of chronic fatigue syndrome patients: Association with signs and symptoms. *Apmis* **2003**, *111*, 557–566. [CrossRef] [PubMed]
47. Staunton, C.; Slokenberga, S.; Mascalzoni, D. The GDPR and the research exemption: Considerations on the necessary safeguards for research biobanks. *Eur. J. Hum. Genet.* **2019**, *27*, 1159–1167. [CrossRef] [PubMed]
48. Allen, M.J.; Powers, M.L.; Gronowski, K.S.; Gronowski, A.M. Human Tissue Ownership and Use in Research: What Laboratorians and Researchers Should Know. *Clin. Chem.* **2010**, *56*, 1675–1682. [CrossRef]
49. van Diest, P.J.; Savulescu, J. For and against: No consent should be needed for using leftover body material for scientific purposes For Against. *BMJ* **2002**, *325*, 648–651. [CrossRef] [PubMed]
50. Health, M. of S.A. and Act on the Medical Use of Human Organs, Tissues and Cells. Available online: https://www.finlex.fi/fi/laki/ajantasa/2001/20010101 (accessed on 20 February 2019).
51. Health, M. of S.A. and Medical Research Act. Available online: https://www.finlex.fi/en/laki/kaannokset/1999/en19990488 (accessed on 20 February 2019).
52. Standardization, I.O. for ISO 15189:2012 Medical Laboratories—Requirements for Quality and Competence. Available online: https://www.iso.org/standard/56115.html (accessed on 20 February 2019).
53. Reed, G.F.; Lynn, F.; Meade, B.D. Use of coefficient of variation in assessing variability of quantitative assays. *Clin. Diagn. Lab. Immunol.* **2002**, *9*, 1235–1239. [CrossRef] [PubMed]
54. Watson, P.F.; Petrie, A. Method agreement analysis: A review of correct methodology. *Theriogenology* **2010**, *73*, 1167–1179. [CrossRef]
55. McHugh, M.L. Interrater reliability: The kappa statistic. *Biochem. Med.* **2012**, *22*, 276–282. [CrossRef]
56. Kim, H.-Y. Statistical notes for clinical researchers: Chi-squared test and Fisher's exact test. *Restor Dent. Endod.* **2017**, *42*, 152. [CrossRef]
57. Kodym, P.; Kurzová, Z.; Berenová, D.; Pícha, D.; Smíšková, D.; Moravcová, L.; Malý, M. Serological Diagnostics of Lyme Borreliosis: Comparison of Universal and Borrelia Species-Specific Tests Based on Whole-Cell and Recombinant Antigens. *J. Clin. Microbiol.* **2018**, *56*, e00601–e006018. [CrossRef]
58. Krause, P.J.; Telford, S.R.; Spielman, A.; Sikand, V.; Ryan, R.; Christianson, D.; Burke, G.; Brassard, P.; Pollack, R.; Peck, J.; et al. Concurrent Lyme disease and babesiosis. Evidence for increased severity and duration of illness. *JAMA* **1996**, *275*, 1657–1660. [CrossRef] [PubMed]
59. Loebermann, M.; Fingerle, V.; Lademann, M.; Fritzsche, C.; Reisinger, E.C. Borrelia burgdorferi and Anaplasma phagocytophilum Coinfection. *Emerg. Infect Dis.* **2006**, *12*, 353–355. [CrossRef] [PubMed]
60. Björsdorff, A.; Wittesjö, B.; Berglund, J.; Massung, R.F.; Eliasson, I. Human Granulocytic Ehrlichiosis as a Common Cause of Tick-associated Fever in Southeast Sweden: Report from a Prospective Clinical Study. *Scand. J. Infect Dis.* **2009**, *34*, 187–191. [CrossRef]
61. Koetsveld, J.; Tijsse-Klasen, E.; Herremans, T.; Hovius, J.W.; Sprong, H. Serological and molecular evidence for spotted fever group Rickettsia and *Borrelia burgdorferi sensu lato* co-infections in The Netherlands. *Ticks Tick-Borne Dis.* **2016**, *7*, 371–377. [CrossRef] [PubMed]
62. Qiu, Y.; Nakao, R.; Ohnuma, A.; Kawamori, F.; Sugimoto, C. Microbial Population Analysis of the Salivary Glands of Ticks; A Possible Strategy for the Surveillance of Bacterial Pathogens. *PLoS ONE* **2014**, *9*, e103961. [CrossRef] [PubMed]
63. Klemola, T.; Sormunen, J.J.; Mojzer, J.; Mäkelä, S.; Vesterinen, E.J. High tick abundance and diversity of tick-borne pathogens in a Finnish city. *Urban Ecosyst.* **2019**, *22*, 817–826. [CrossRef]
64. Junttila, J.; Tanskanen, R.; Tuomi, J. Prevalence of Borrelia burgdorferi in selected tick populations in Finland. *Scand J. Infect Dis* **1994**, *26*, 349–355. [CrossRef]
65. Haapasalo, K.; Suomalainen, P.; Sukura, A.; Siikamaki, H.; Jokiranta, T.S. Fatal babesiosis in man, Finland, 2004. *Emerg. Infect Dis.* **2010**, *16*, 1116–1118. [CrossRef] [PubMed]
66. Steere, A.C.; Dhar, A.; Hernandez, J.; Fischer, P.A.; Sikand, V.K.; Schoen, R.T.; Nowakowski, J.; McHugh, G.; Persing, D.H. Systemic symptoms without erythema migrans as the presenting picture of early Lyme disease. *Am. J. Med.* **2003**, *114*, 58–62. [CrossRef]
67. Nowakowski, J.; Schwartz, I.; Liveris, D.; Wang, G.; Aguero-Rosenfeld, M.E.; Girao, G.; McKenna, D.; Nadelman, R.B.; Cavaliere, L.F.; Wormser, G.P.; et al. Laboratory diagnostic techniques for patients with early Lyme disease associated with erythema migrans: A comparison of different techniques. *Clin. Infect. Dis.* **2001**, *33*, 2023–2027. [CrossRef] [PubMed]

68. Belongia, E.A.; Reed, K.D.; Mitchell, P.D.; Mueller-Rizner, N.; Vandermause, M.; Finkel, M.F.; Kazmierczak, J.J. Tickborne Infections as a Cause of Nonspecific Febrile Illness in Wisconsin. *Clin. Infect. Dis.* **2001**, *32*, 1434–1439. [CrossRef] [PubMed]
69. Andreasson, U.; Perret-Liaudet, A.; van Doorn, L.J.; Blennow, K.; Chiasserini, D.; Engelborghs, S.; Fladby, T.; Genc, S.; Kruse, N.; Kuiperij, B.H.; et al. A Practical Guide to Immunoassay Method Validation. *Front. Neurol.* **2015**, *6*, 179. [CrossRef]
70. Crowther, J.R. *The ELISA Guidebook*; Humana Press Inc.: Totowa, NJ, USA, 2001; Volume 149.
71. Meriläinen, L.; Herranen, A.; Schwarzbach, A.; Gilbert, L. Morphological and biochemical features of Borrelia burgdorferi pleomorphic forms. *Microbiology* **2015**, *161*, 516–527. [CrossRef] [PubMed]
72. Meriläinen, L.; Brander, H.; Herranen, A.; Schwarzbach, A.; Gilbert, L. Pleomorphic forms of Borrelia burgdorferi induce distinct immune responses. *Microb Infect* **2016**, *18*, 484–495. [CrossRef]
73. Diterich, I.; Rauter, C.; Kirschning, C.J.; Hartung, T. Borrelia burgdorferi-induced tolerance as a model of persistence via immunosuppression. *Infect Immun.* **2003**, *71*, 3979–3987. [CrossRef]
74. Tuuminen, T.; Hedman, K.; Söderlund-Venermo, M.; Seppälä, I. Acute Parvovirus B19 Infection Causes Nonspecificity Frequently in Borrelia and Less Often in Salmonella and Campylobacter Serology, Posing a Problem in Diagnosis of Infectious Arthropathy. *Clin. Vaccine Immunol.* **2011**, *18*, 167–172. [CrossRef] [PubMed]

Te?ted Oy, Mattilanniemi 6-8, 40100 Jyväskylä, Finland.

TICKPLEX® PLUS INSTRUCTIONS FOR USE

IVD CE

1. TITLE: TICKPLEX® PLUS. A complete diagnostic kit for tick-borne disease pathogens.

2. INTENDED USE

The TICKPLEX® PLUS test provides a quantitative and qualitative *in vitro* assay for human IgM and IgG antibodies against multiple microbial antigens in human serum or plasma. The kit tests for *Borrelia afzelii*, *Borrelia burgdorferi,* and *Borrelia garinii* infections and the tests include persistent antigens of the different Borrelia species. In addition, the kit tests for co-infections (*Babesia microti, Bartonella henselae*, *Ehrlichia chaffeensis, Rickettsia akari)*, and opportunistic infections (Coxsackievirus*,* Epstein-Barr virus, Human parvovirus B19*, Mycoplasma fermentans* and *Mycoplasma pneumoniae*) associated with tick-borne diseases. This kit is intended to aid in diagnosis of tick-borne pathogen infections. The test kit and TICKPEX® PLUS analyze excel sheet are intended to aid in the diagnosis of diseases associated with a tick bite. The test kit and TICKPEX® PLUS analyze excel sheet are for professional use only in clinical laboratory environment and are not to be used for self-testing.

3. TEST PRINCIPLE

TICKPLEX® PLUS is an indirect enzyme linked immunosorbent assay (ELISA). Micro wells on TICKPLEX® PLUS ELISA plates have been coated with inactivated whole cell lysate or peptide antigens. Antigen (A1 to A10) coating order for TICKPLEX® PLUS can be noted from TICKPLEX® PLUS plate layout in section 7. Antibodies that are specific against antigens being tested in TICKPLEX® PLUS will bind to the immobilized antigen(s) on the plate. Antibodies that do not bind with antigens are washed away. However, antibodies that form complexes with the antigen(s) can be recognized by anti-human IgM and anti-human IgG antibodies. Anti-human IgM/IgG are conjugated with horseradish peroxidase (HRP). Presence of conjugated anti-human IgM/IgG can be seen by an enzymatic reaction with 3,3',5,5'-tetramethylbenzidine (chromogenic substrate). Human antibodies that is negative against the antigens being tested in TICKPLEX® PLUS will not form complexes with immobilized antigens. A mild change in the chromogenic substrate color due to a reaction with a negative human antibody can be differentiated from a reaction with a borderline, and positive human antibody binding.

4. TICKPLEX® PLUS ELISA KIT COMPONENTS AND STORAGE

Table 1. List of TICKPLEX® PLUS ELISA kit components, quantities provided in the kit, and storage conditions.

ID	TICKPLEX® PLUS ELISA kit components	Quantity	Storage upon receipt
A	ELISA microplate coated with inactivated microbial antigens	10 plates	+2°C to +8°C
B	Wash buffer concentrate (25X phosphate buffer saline)	2 x 125 ml	+2°C to +8°C
C	Sample buffer concentrate (2% bovine serum albumin)	150 ml	+2°C to +8°C
D	100 % IgM serum diluent concentrate	50 ml	+2°C to +8°C
E	Enzyme substrate solution [3,3',5,5'-tetramethylbenzidine (TMB)]	125 ml	+2°C to +8°C
F	Stop solution [2 M Sulphuric acid (H_2SO_4)]	100 ml	+2°C to +8°C
G	Anti-human IgM conjugated with HRP	60 ml	+2°C to +8°C
H	Anti-human IgG conjugated with HRP	60 ml	+2°C to +8°C
I	Negative serum control (TEZ1) with SDS document	25 ml	+2°C to +8°C
J	TICKPLEX PLUS ANALYZE (sent via email)	1	N/A
K	TICKPLEX PLUS Quality control certificate	1 document	N/A

Note: Please inspect that all kit components are available in the kit and packed as specified.

Version 18: 07/02/2022

Note: Please check that you have received Analyze Excel Sheet with matching LOT information marked on Containers 1 and 2.

Note: Please check that Analyze Excel Sheet opens up in your computer. Instructions how to use software for test result calculations are included as part of program.

5. MATERIALS REQUIRED BUT NOT PROVIDED WITH THE KIT

i. Distilled/deionized water for dilution of wash buffer concentrate.
ii. Appropriate equipment for pipetting, liquid dispensing, and washing ELISA microplate.
iii. Spectrophotometer/colorimeter/microplate reader. Absorbance wavelength required = 450 nm with bottom reading.
iv. Sixty (60) 2 ml Eppendorf tubes.
v. Waste for safe disposal of potentially infectious material (example; human serum sample).

6. REAGENT AND SERUM SAMPLE PREPARATIONS FOR PERFORMING ONE PLATE

A TICKPLEX® PLUS ELISA microplate can test negative serum control (TEZ1) and three patients. The following kit components have been provided in ready to use format: the ELISA microplates coated with inactivated microbial antigens, enzyme substrate solution [3,3',5,5'-tetramethylbenzidine (TMB)], and stop solution [2 M Sulphuric acid (H_2SO_4)].

Follow instructions below to dilute the remaining kit components to perform one (1) TICKPLEX® PLUS microplate:

i. Diluting buffer solution

- **Wash buffer concentrate dilution** → Add 300 ml of distilled / deionized water to 12.5 ml wash buffer concentrate (kit component B) to obtain **1X wash buffer**.
- **Sample buffer concentrate dilution** → Add 15 ml of 1X wash buffer to 15 ml sample buffer concentrate (kit component C) to obtain **1 % sample buffer**.
- **100 % IgM serum diluent concentrate dilution** → Add 2.5 ml of 1 % sample buffer to 2.5 ml of 100 % IgM serum diluent (kit component D) to obtain **50 % IgM serum diluent**.

Storage after dilution:
Store 1X wash buffer and 1 % sample buffer at +2°C to +8°C for maximum 6 weeks.
50 % IgM serum diluent should be used immediately after preparation.
CAUTION: Negative serum control is a human serum sample which is classified as non-hazardous substance. This human serum has been verified as negative for the most relevant infections, but it is not a complete assurance that infectious agents are absent. Thus, the user should handle this product as if capable of transmitting infection.

ii. Diluting patient serum sample

A TICKPLEX® PLUS ELISA microplate can test three (3) patients. To test one patient at 1:200 dilution, 1 ml of diluted sera for IgM testing and 1 ml of diluted sera for IgG testing are needed.

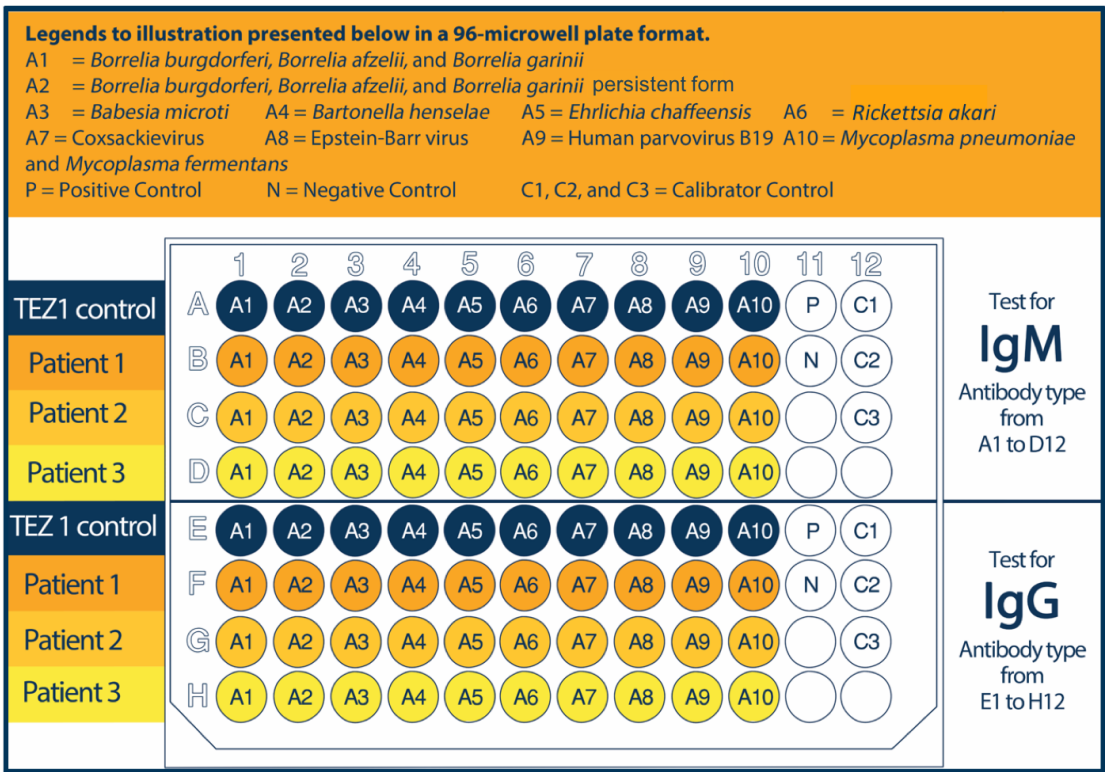
- **For IgM testing** → add 5 µl of patient sera sample to 995 µl of **50 % IgM serum diluent.**

- **For IgG testing** → add 5 µl of patient sera sample to 995 µl of **1 % sample buffer**

7. DIRECTIONS TO PERFORM THE TICKPLEX® PLUS ELISA

To perform TICKPLEX® PLUS, the plate layout provided below and the instructions in this section must be followed. Read the instructions carefully before performing the test.

TICKPLEX® PLUS PLATE LAYOUT



i. Negative serum control (TEZ1) and patient sample addition and incubation

- Place the reagents and plate at room temperature (+15°C to +25°C) for 30 min before performing the test.
- Unwrap the TICKPLEX® PLUS ELISA microplate from its vacuum packing.
- Pipetting order for **IgM** test is as follows:
    - Pipette 100 µl of negative serum control (TEZ1) in wells A1 to A10.
    - Pipette 100 µl of patient 1 serum sample freshly diluted in 50 % IgM serum diluent in wells B1 to B10 each.
    - Pipette 100 µl of patient 2 serum sample freshly diluted in 50 % IgM serum diluent in wells C1 to C10 each.
    - Pipette 100 µl of patient 3 serum sample freshly diluted in 50 % IgM serum diluent in wells D1 to D10 each.
- Pipetting order for **IgG** test is as follows:
    - Pipette 100 µl of negative serum control (TEZ1) in wells E1 to E10 each.
    - Pipette 100 µl of patient 1 serum sample freshly diluted in 1 % sample buffer in wells F1 to F10 each.
    - Pipette 100 µl of patient 2 serum sample freshly diluted in 1 % sample buffer in wells G1 to G10 each.
    - Pipette 100 µl of patient 3 serum sample freshly diluted in 1 % sample buffer in wells H1 to H10 each.

- Pipette 100 µl of 1 % sample buffer to IgM and IgG positive control (P), negative control (N), and calibrator (C1, C2, and C3) wells.
- Cover the plate with its lid and incubate for 1 hour at room temperature.

ii. Anti-human IgM and IgG HRP conjugated antibodies addition and incubation

- **Wash step** → Dispense and aspirate 200 µl of 1X wash buffer five times in all wells.
- After washing, thoroughly dispose of all liquid from the microplate by tapping it on an absorbent paper with the openings facing downwards to remove all residual wash buffer.

IMPORTANT: Anti-human IgM and IgG conjugated with HRP dilution should be gently mixed before use (example; 1 ml single-channel pipette).

- In each well, pipette 100 µl of anti-human **IgM** conjugated with HRP in rows A, B, C, and D.
- In each well, pipette 100 µl of anti-human **IgG** conjugated with HRP in rows E, F, G, and H.
- Cover the plate and incubate for 1 hour at room temperature.

iii. Enzyme substrate solution addition and incubation

- **Wash step** → Dispense and aspirate 200 µl of 1X wash buffer five times in all wells.
- After washing, thoroughly dispose of all liquid from the microplate by tapping it on an absorbent paper with the openings facing downwards to remove all residual wash buffer.

IMPORTANT: Enzyme substrate solution (TMB) must reach room temperature before use. The enzyme substrate solution must be clear to use. DO NOT USE if the solution is colored.

- Add 100 µl of enzyme substrate solution (TMB) in all wells.
- Cover the plate and incubate in the dark (example, covering with aluminum foil) at room temperature.
- Incubate plate for a total of **30 min** at room temperature.

iv. Stop solution addition and plate reading at 450 nm

- Add **100 µl of** stop solution (H_2SO_4) in all well.
- Read the optical absorbance immediately at 450 nm (bottom reading).
- Resulting optical density (OD) values will be used to calculate patient 1, 2, and 3 responses to TICKPLEX® PLUS antigens.

8. CALCULATION OF RESULTS

For manual calculations, the quality control certificate provided with the test kit and the plate layout provided above must be followed along with the instructions in this section.

i. Optical density index (ODI) calculations

- **Evaluate validity of the test performed for ODI analysis**
  The test performed is valid if;
  - The OD values for IgM (well A11) and IgG (well E11) **positive controls are ≥ 1.0**

- The OD values for IgM (well B11) and IgG (well F11) **negative controls are ≤ 0.5**

- **Calculate individual cut-off values for antigens A1 to A10**
  - **For IgM test result →** Insert optical density values from wells A1 to A10 in table 2 (last row named "Tested results") provided in the quality control certificate.
  - **For IgG test result →** Insert optical density values from wells E1 to E10 in table 3 (last row named "Tested results") provided in the quality control certificate.
  - For each antigen in tables 2 and 3 (in quality control certificate), calculate the mean.
  - Multiply each IgM and IgG mean with the correction factor provided in table 4 of the quality control certificate to obtain cut-off values.

- **Calculate the ODI**
  - For each antigen (A1 to A10) and patient (patient 1, 2, and 3), divide the OD value over its cut off value calculated above.
    For IgM and IgG, calculate the ODI according to the following formula,

$$\frac{\textbf{OD value of a patient for antigen A\#}}{\textbf{Cut off value of antigen A\#}} = \textbf{ODI}$$

    where, A# = Antigen A1, A2, A3, A4, A5, A6, A7, A8, A9, or A10.

    Interpret results as follows,

ODI < 0.9	**NEGATIVE**
ODI ≥ 0.9 to < 1.0	**BORDERLINE**
ODI ≥ 1	**POSITIVE**

ii. Antibody titer calculations

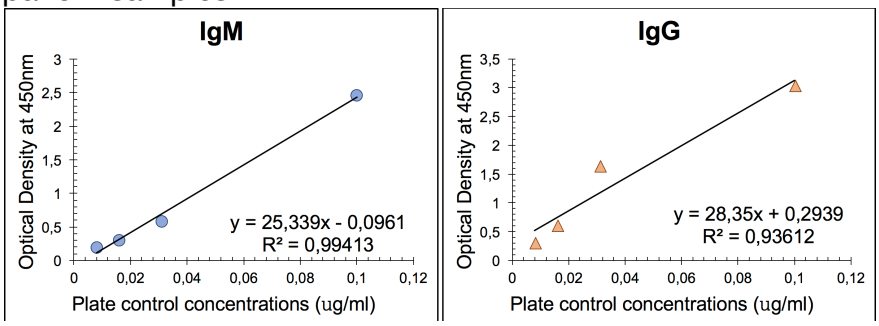
- **Create a standard curve for IgM and IgG**
  - Use a computer to create a scatter plot with OD-values of positive, calibrators 1, 2, and 3 controls on y-axis and their corresponding concentrations on x-axis in the same order as presented below in Table 2.

Table 2. Positive, calibrators 1, 2, and 3 controls concentrations (in µg) for the standard curve.

Controls required for creating a standard curve	Reference well number		Concentration (µg/ml)
	IgM	IgG	
Calibrator 3	C12	G12	0.008
Calibrator 2	B12	F12	0.016
Calibrator 1	A12	E12	0.031
Positive control	A11	E11	0.1

  - Add a linear trend line with straight-line equation and R-squared (R^2) value to the scatter plot.

The following plots are an example of a typical standard curve. DO NOT use the standard curves below to determine antibody concentrations in patient samples.



- **Evaluate validity of the test performed for antibody titer analysis**
  The test is valid if,
  - The **R^2 value is ≥ 0.75** for IgM and IgG.

- **Calculate antibody concentrations in patient samples**
  Use the straight-line equation ($y = mx \pm c$) from IgM and IgG standard curves.
  - In place of "y", insert OD value of an antigen for a patient and solve for "x". Resulting value for "x" is the antibody concentration in µg/ml.

iii. Test result interpretation

It is recommended that both ODI and corresponding antibody titer values are taken into clinical consideration. A negative immune response on TICKPLEX® PLUS does not preclude the possibility of a positive immune response to microbes associated with tick-borne disease: false negative immune responses can be due to stage of infection (e.g., specimen obtained prior to the development of cellular immune response), co-morbid conditions which affect immune functions, incorrect handling of the blood collection tubes following venipuncture, incorrect performance of the assay, or other immunological variables. Patients in early stages of infection may not produce detectable levels of antibody. Further, early antibiotic therapy after ECM (*erythema chronicum migrans*) may diminish or abrogate good antibody response). Immunocompromised patients may never generate detectable antibody levels.
A positive immune response on TICKPLEX® PLUS result should not be the sole or definitive basis for determining infection with tick-borne pathogen. Incorrect performance of the assay may cause false-positive responses. A positive immune response on TICKPLEX® PLUS should be followed by further medical evaluation and diagnostic evaluation for an active tick-borne disease. Sera/plasma from patients with other spirochetal diseases (syphilis, yaws, pinta, leptospirosis and relapsing fever), infectious mononucleosis or systemic lupus erythematosus may give false positive results.
The IgM antibody test can be used for asymptomatic patients a minimum of 2 weeks after confirmed tick bite.
The IgG antibody test can be used for asymptomatic patients a minimum of 4 weeks after confirmed tick bite.
The IgM antibody test can be used for tick-borne diseases symptomatic patients a minimum of 2 weeks after confirmed tick bite.
The IgG antibody test can be used for tick-borne diseases symptomatic patients a minimum of 4 weeks after confirmed tick bite.

9. CHARACTERISTICS OF THE TEST

i. Validity of the test

The test is intended for determination of specific IgM and IgG antibody responses in human serum or plasma. Te?ted Oy does not guarantee kit operation with EDTA plasma, heparin plasma, citrate plasma, or hemolytic serum/plasma. The test can be performed on minimum 4 ml of whole drawn blood, on samples stored generally at +4°C for up to 14 days, on diluted samples within one working day, and on samples stored at -20°C for up to one year.

ii. Clinical studies

Figure 1 demonstrates the clinical relevance of TICKPLEX® PLUS antigens using the IgM and IgG immune responses from several patient categories. Following guidelines from the Centers for Disease Control and Prevention (CDC) two-tier criteria and the Infectious Disease Society of America (IDSA), we categorized specimens into CDC acute, CDC late, CDC negative, and Post-Treatment Lyme Disease Syndrome (PTLDS). Further, the study included immunocompromised patients who suffered from flu-like symptoms or late Lyme disease symptoms, low CD57 cell count and tested negative by lymphocyte cells against Borrelia antigens. Likewise, the unspecific patient category included individuals with flu-like signs or late Lyme disease symptoms but no laboratory tests for tick-borne disease. Lastly, the healthy group comprised specimens without flu-like signs or late Lyme disease symptoms, negative IgM or IgG serology utilizing the CDC two-tier LD diagnosis criteria, and healthy donors.

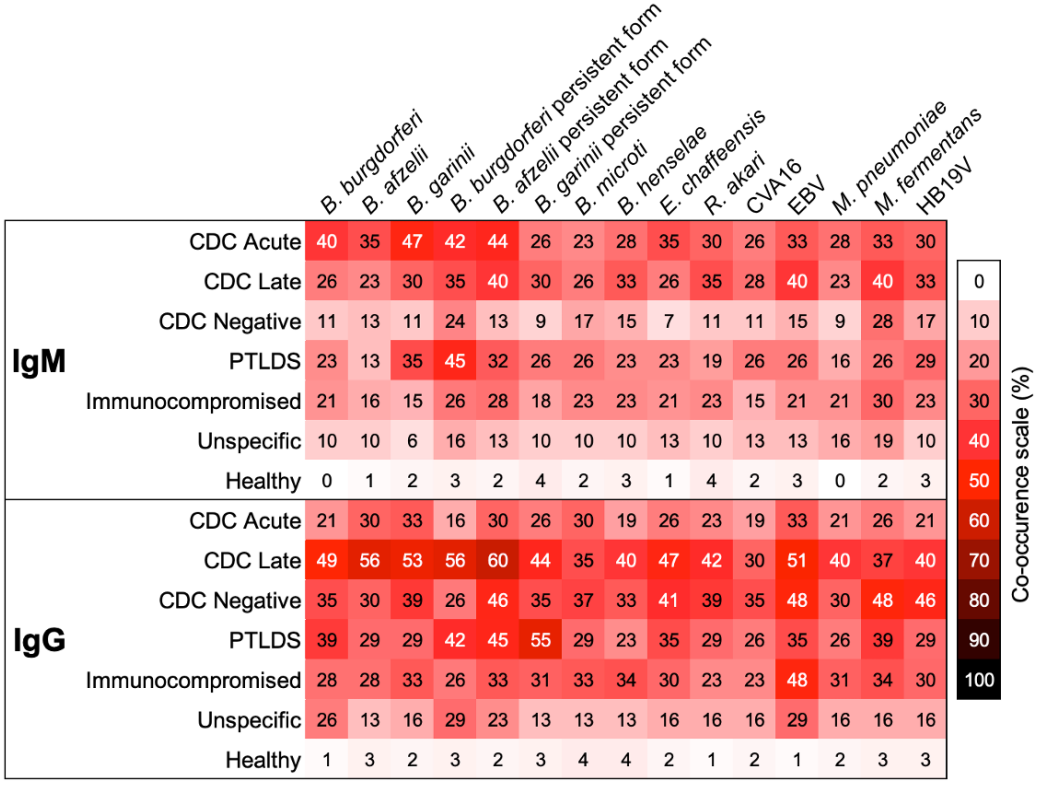


Figure 1. IgM and IgG immune responses by individual patient categories to TICKPLEX® PLUS antigens. Patient categories refer to individuals from the Centers for Disease Control and Prevention (CDC) acute, CDC late, CDC negative, Post-Treatment Lyme Disease Syndrome (PTLDS), immunocompromised, and unspecific. Microbes include *Borrelia burgdorferi sensu stricto*, *Borrelia afzelii*, *Borrelia garinii*, *Borrelia burgdorferi sensu stricto* persistent form, *Borrelia afzelii* persistent form, *Borrelia garinii* persistent form, *Babesia microti*, *Bartonella henselae*, *Ehrlichia*

chaffeensis, *Rickettsia akari*, Coxsackievirus A16 (CVA16), Epstein-Barr virus (EBV), *Mycoplasma pneumoniae*, *Mycoplasma fermentans*, and Human parvovirus B19 (HB19V).

iii. Diagnostic sensitivity and specificity

Table 3. The diagnostic sensitivity, specificity, and their evaluation has been tabulated.

Antigen	IgM/IgG Sensitivity	IgM/IgG Specificity	Evaluation criteria
Borrelia species and persistent Borrelia forms	95%	98%	Previous clinical diagnostic results for 331 patients with Lyme disease were compared with test results from the Tickplex study performed at the Jyväskylä University.
Babesia microti	100%	80%	Previous clinical diagnostic results for 200 patients with *Babesia microti* were compared with test results from the Tickplex study performed at the Jyväskylä University.
Bartonella henselae	80%	100%	Previous clinical diagnostic results for 206 patients with *Bartonella henselae* were compared with test results from the Tickplex study performed at the Jyväskylä University.
Ehrlichia chaffeensis	94%	90%	Previous clinical diagnostic results for 204 patients with *Ehrlichia chaffeensis* were compared with test results from the Tickplex study performed at the Jyväskylä University.
Rickettsia akari	100%	100%	Previous clinical diagnostic results for 187 patients with Lyme disease were tested in the Tickplex study performed at Jyväskylä University. Results from Tickplex study were compared with test results from the verification study at Te?ted Oy.
Coxsackievirus	90%	100%	Previous clinical diagnostic results for 182 patients with Coxsackievirus were compared with test results from the Tickplex study performed at the Jyväskylä University.
Epstein-Barr virus	100%	100%	Previous clinical diagnostic results for 188 patients with Epstein-Barr virus were compared with test results from the Tickplex study performed at the Jyväskylä University.
Human parvovirus B19	86%	100%	Previous clinical diagnostic results for 178 patients with Human parvovirus B19 were compared with test results from the Tickplex study performed at the Jyväskylä University.
Mycoplasma pneumoniae and *Mycoplasma fermentans*	100%	100%	Previous clinical diagnostic results for 187 patients with Lyme disease were tested in the Tickplex study performed at Jyvaskyla University. Results from Tickplex study were compared with test results from the verification study at Te?ted Oy.

10. SAFETY PRECAUTIONS

i. For *in vitro* diagnostic use only.

ii. When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. Do not smoke, drink, or eat while performing or preparing for the assay. Do not pipette by mouth. Wear disposable gloves while handling reagents or samples and wash your hands thoroughly afterwards. Avoid spilling or producing aerosols.

iii. Do not use kit if any reagent bottle or the microplate shows signs of damage or leakage prior to use. Do not use blood collection tubes or ELISA kit after the expiration date.

iv. Ensure that laboratory equipment such as plate washers and readers have been calibrated / validated before use.

v. Handle stop solution (H_2SO_4) with care. Avoid contact with skin or mucous membranes. In case of contact with skin, rinse immediately with plenty of water and seek for medical advice. Liquid waste containing acid [stop solution (H_2SO_4)] should be neutralized in 4% sodium bicarbonate solution.

vi. Handle human blood as if potentially infectious. Observe relevant blood handling guidelines. While preparing and perform the assay, all material that encountered human serum should be considered as contagious and therefore should be handled / disposed according to appropriate regulations.

vii. If not using the complete solutions at one time, use in sterile conditions to prevent contamination.

11. HANDLING PRECAUTIONS

i. Follow assay procedure indicated above.
ii. Avoid microbial contamination of serum samples and kit reagents.
iii. If not using the complete solutions at one time, use sterile conditions to prevent contamination.
iv. Avoid cross-contamination of reagents.
v. Avoid contact of enzyme substrate solution (TMB) with oxidizing agents, metal surfaces, and light.
vi. Enzyme substrate solution (TMB) must be clear on use; do not use if the solution is colored.
vii. Store reagents in conditions mentioned above when not in use.
viii. Do not re-use diluted human antibodies or IgM/IgG conjugated with HRP samples in 1% sample buffer. Always prepare fresh.
ix. Do not use reagents after the expiry date printed on the label.
x. Variations in the test results are usually due to:
    - Insufficient mixing of reagents and samples
    - Inaccurate pipetting and inadequate incubation times
    - Poor washing techniques or spilling the rim of well with sample or IgM/IgG conjugated with HRP
    - Use of identical pipette tips for different solutions
    - Use of contaminated pipette tips
    - The ELISA microplate and assay reagents such as enzyme substrate solution (TMB) did not reach room temperature +15°C to +25°.

Technical Assistance: info@tezted.com More information: www.tezted.com
UDI-DI product identifier: UDI-DI: 111745456742 (Tickplex Plus) Classification B.

Te?ted Oy, Mattilanniemi 6-8, 40100 Jyväskylä, Finland.

TICKPLEX® BASIC INSTRUCTIONS FOR USE

IVD CE

1. TITLE: TICKPLEX® BASIC. A complete diagnostic kit for tick-borne disease pathogens.

2. INTENDED USE

The TICKPLEX® BASIC test provides a quantitative and qualitative *in vitro* assay for human IgM and IgG antibodies against multiple microbial antigens in human serum or plasma. The kit tests for *Borrelia afzelii*, *Borrelia burgdorferi,* and *Borrelia garinii* infections and the tests include persistent antigens of the different borrelia species. The test kit and TICKPEX® BASIC analyze excel sheet are intended to aid in the diagnosis of tick-borne diseases. The test kit and TICKPEX® BASIC analyze excel sheet are for professional use only in clinical laboratory environment and are not to be used for self-testing.

3. TEST PRINCIPLE

TICKPLEX® BASIC is an indirect enzyme linked immunosorbent assay (ELISA). Micro wells on TICKPLEX® BASIC ELISA plates have been coated with inactivated whole cell lysate or peptide antigens. Antigens (A1 and A2) coating order for TICKPLEX® BASIC can be noted from TICKPLEX® BASIC plate layout in section 7. Antibodies that are specific against antigens being tested in TICKPLEX® BASIC will bind to the immobilized antigen(s) on the plate. Antibodies that do not bind with antigens are washed away. However, antibodies that form complexes with the antigen(s) can be recognized by anti-human IgM and anti-human IgG antibodies. Anti-human IgM/IgG are conjugated with horseradish peroxidase (HRP). Presence of conjugated anti-human IgM/IgG can be seen by an enzymatic reaction with 3,3',5,5'-tetramethylbenzidine (chromogenic substrate). Human antibodies that is negative against the antigens being tested in TICKPLEX® BASIC will not form complexes with immobilized antigens. A mild change in the chromogenic substrate color due to a reaction with a negative human antibody can be differentiated from a reaction with a borderline, and positive human antibody binding.

4. TICKPLEX® BASIC ELISA KIT COMPONENTS AND STORAGE

Table 1. List of TICKPLEX® BASIC ELISA kit components, quantities provided in the kit, and storage conditions.

ID	TICKPLEX® BASIC ELISA kit components	Quantity	Storage upon receipt
A	ELISA microplate coated with inactivated microbial antigens	10 plates	+2°C to +8°C
B	Wash buffer concentrate (25X phosphate buffer saline)	2 x 125 ml	+2°C to +8°C
C	Sample buffer concentrate (2% bovine serum albumin)	150 ml	+2°C to +8°C
D	100 % IgM serum diluent concentrate	50 ml	+2°C to +8°C
E	Enzyme substrate solution [3,3',5,5'-tetramethylbenzidine (TMB)]	125 ml	+2°C to +8°C
F	Stop solution [2 M Sulphuric acid (H_2SO_4)]	100 ml	+2°C to +8°C
G	Anti-human IgM conjugated with HRP	60 ml	+2°C to +8°C
H	Anti-human IgG conjugated with HRP	60 ml	+2°C to +8°C
I	TICKPLEX BASIC ANALYZE (sent via email)	1	N/A
J	TICKPLEX BASIC Quality control certificate	1 document	N/A

Note: Please inspect that all kit components are available in the kit and packed as specified.

5. MATERIALS REQUIRED BUT NOT PROVIDED WITH THE KIT

i. Distilled/deionized water for dilution of wash buffer concentrate and sample buffer concentrate.
ii. Appropriate equipment for pipetting, liquid dispensing, and washing ELISA microplates.
iii. Spectrophotometer/colorimeter/microplate reader. Absorbance wavelength required = 450 nm.
iv. Five hundred (500) 2 ml Eppendorf tubes.
v. Waste for safe disposal of potentially infectious material (example; human serum sample).

6. REAGENT AND SERUM SAMPLE PREPARATIONS REQUIRED

A TICKPLEX® BASIC ELISA microplate can test twenty patients. The following kit components have been provided in ready to use format: the ELISA microplates coated with inactivated microbial antigens, enzyme substrate solution [3,3',5,5'-tetramethylbenzidine (TMB)], and stop solution [2 M Sulphuric acid (H_2SO_4)].

Follow the instructions below to dilute the remaining kit components to perform one (1) TICKPLEX® BASIC microplate:

i. Diluting buffer solution

- **Wash buffer concentrate dilution →** Add 300 ml of distilled / deionized water to 12.5 ml wash buffer concentrate (kit component B) to obtain **1X wash buffer**.
- **Sample buffer concentrate dilution →** Add 15 ml of 1X wash buffer to 15 ml sample buffer concentrate (kit component C) to obtain **1 % sample buffer**.
- **100 % IgM serum diluent concentrate dilution →** Add 2.5 ml of 1 % sample buffer to 2.5 ml of 100 % IgM serum diluent (kit component D) to obtain **50 % IgM serum diluent**.

Storage after dilution:
Store 1X wash buffer and 1 % sample buffer at +2°C to +8°C for maximum 6 weeks.
50 IgM serum diluent should be used immediately after preparation.

ii. Diluting patient serum sample

A TICKPLEX® BASIC ELISA microplate can test twenty (20) patients. To test one patient at 1:200 dilution, 200 µl of diluted sera for IgM testing and 200 µl of diluted sera for IgG testing are needed.

- **For IgM testing →** add 1 µl of patient sera sample to 199 µl of **50 % IgM serum diluent**
- **For IgG testing →** add 1 µl of patient sera sample to 199 µl of **1 % sample buffer**

IMPORTANT: Anti-human IgM and IgG conjugated with HRP antibodies should be thoroughly mixed before use (example; vortex-mixer).

7. DIRECTIONS TO PERFORM THE TICKPLEX® BASIC ELISA

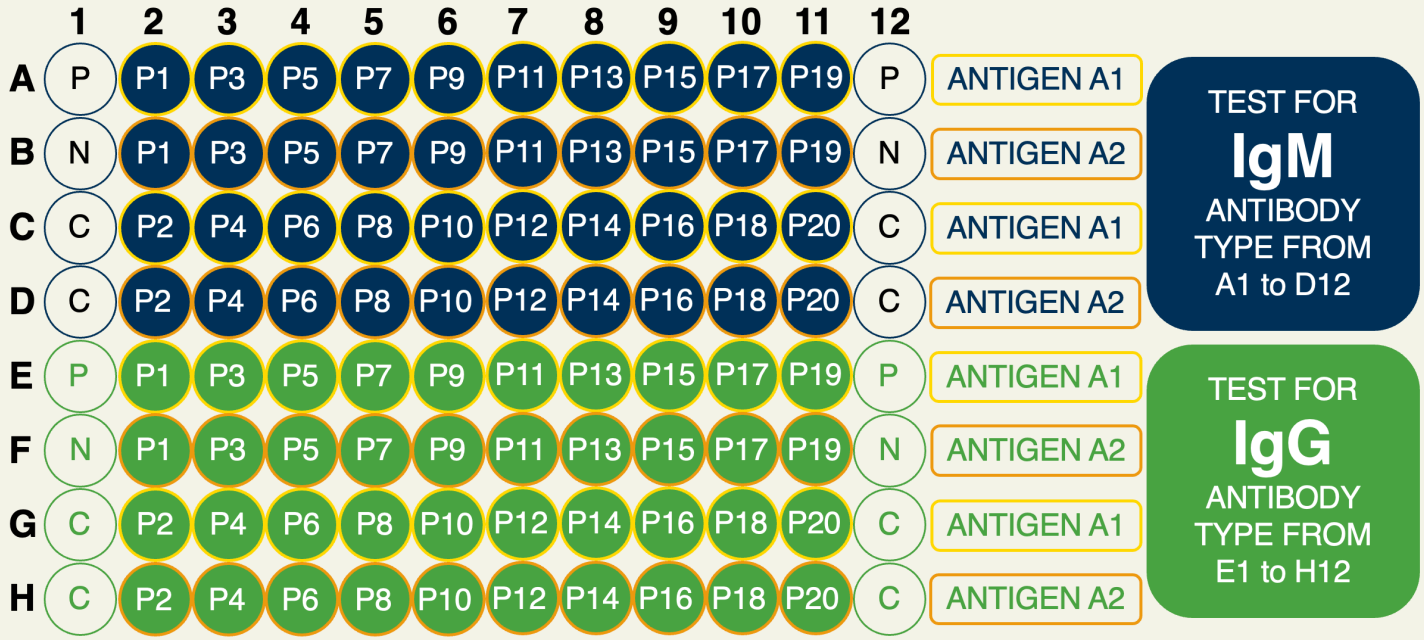
To perform TICKPLEX® BASIC, the plate layout provided below, and the instructions in this section must be followed. Read the instructions carefully before performing the test.

TICKPLEX® BASIC PLATE LAYOUT

Legends to illustrations presented below in a 96-microwell plate format

P = positive plate control; **N** = negative plate control; **C** = calibrator control
P1 to P20 = wells to add diluted serum samples of patients 1 to 20

NOTE: Columns 1 and 12 contain IgM and IgG plate controls. Only one plate control strip is required to perform the assay.
IMPORTANT: Always place a plate control strip in column 1 when performing the assay. Carefully seal and refrigerate unused strips immediately.



NOTE: Antigen A1 includes *Borrelia burgdorferi, Borrelia afzelii, and Borrelia garinii*; antigen A2 includes *Borrelia burgdorferi, Borrelia afzelii, and Borrelia garinii* in persisted form

i. Patient sample addition and incubation

- Place the reagents and plate at room temperature (+15°C to +25°C) for 30 min before performing the test.
  NOTE: Do not open the bag until the wrapping and its content have reached room temperature to prevent condensation.

- Unwrap the TICKPLEX® BASIC ELISA microplate from its vacuum packing.
- Always place a plate control strip (with a red dot) in column 1 when performing the assay. Carefully seal and refrigerate unused strips immediately.
- Pipette 100 µl of 1 % sample buffer to IgM and IgG positive control (P), negative control (N), and calibrator (C) wells in column 1.
- Pipetting order for the **twenty patient serum samples freshly diluted in 50 % IgM serum diluent for the IgM test** is as follows:
  1. **Column 2**, pipette 100 µl of serum sample of patient number 1 (**P #1**) to wells A2 and B2, and patient number 2 (**P #2**) to wells C2 and D2.
  2. **Column 3**, pipette 100 µl of serum sample of patient number 3 (**P #3**) to wells A3 and B3, and patient number 4 (**P #4**) to wells C3 and D3.
  3. **Column 4**, pipette 100 µl of serum sample of patient number 5 (**P #5**) to wells A4 and B4, and patient number 6 (**P #6**) to wells C4 and D4.
  4. **Column 5**, pipette 100 µl of serum sample of patient number 7 (**P #7**) to wells A5 and B5, and patient number 8 (**P #8**) to wells C5 and D5.

5. **Column 6**, pipette 100 µl of serum sample of patient number 9 (**P #9**) to wells A6 and B6, and patient number 10 (**P #10**) to wells C6 and D6.
6. **Column 7**, pipette 100 µl of serum sample of patient number 11 (**P #11**) to wells A7 and B7, and patient number 12 (**P #12**) to wells C7 and D7.
7. **Column 8**, pipette 100 µl of serum sample of patient number 13 (**P #13**) to wells A8 and B8, and patient number 14 (**P #14**) to wells C8 and D8.
8. **Column 9**, pipette 100 µl of serum sample of patient number 15 (**P #15**) to wells A9 and B9, and patient number 16 (**P #16**) to wells C9 and D9.
9. **Column 10**, pipette 100 µl of serum sample of patient number 17 (**P #17**) to wells A10 and B10, and patient number 18 (**P #18**) to wells C10 and D10.
10. **Column 11**, pipette 100 µl of serum sample of patient number 19 (**P #19**) to wells A11 and B11, and patient number 20 (**P #20**) to wells C11 and D11.

- Pipetting order for the **twenty patient serum samples freshly diluted in 1% sample buffer for IgG test** is as follows:
    1. **Column 2**, pipette 100 µl of serum sample of patient number 1 (**P #1**) to wells E2 and F2, and patient number 2 (**P #2**) to wells G2 and H2.
    2. **Column 3**, pipette 100 µl of serum sample of patient number 3 (**P #3**) to wells E3 and F3, and patient number 4 (**P #4**) to wells G3 and H3.
    3. **Column 4**, pipette 100 µl of serum sample of patient number 5 (**P #5**) to wells E4 and F4, and patient number 6 (**P #6**) to wells G4 and H4.
    4. **Column 5**, pipette 100 µl of serum sample of patient number 7 (**P #7**) to wells E5 and F5, and patient number 8 (**P #8**) to wells G5 and H5.
    5. **Column 6**, pipette 100 µl of serum sample of patient number 9 (**P #9**) to wells E6 and F6, and patient number 10 (**P #10**) to wells G6 and H6.
    6. **Column 7**, pipette 100 µl of serum sample of patient number 11 (**P #11**) to wells E7 and F7, and patient number 12 (**P #12**) to wells G7 and H7.
    7. **Column 8**, pipette 100 µl of serum sample of patient number 13 (**P #13**) to wells E8 and F8, and patient number 14 (**P #14**) to wells G8 and H8.
    8. **Column 9**, pipette 100 µl of serum sample of patient number 15 (**P #15**) to wells E9 and F9, and patient number 16 (**P #16**) to wells G9 and H9.
    9. **Column 10**, pipette 100 µl of serum sample of patient number 17 (**P #17**) to wells E10 and F10, and patient number 18 (**P #18**) to wells G10 and H10.
    10. **Column 11**, pipette 100 µl of serum sample of patient number 19 (**P #19**) to wells E11 and F11, and patient number 20 (**P #20**) to wells G11 and H11.

- Cover the plate with its lid and incubate for 1 hour at room temperature.

ii. Anti-human IgM and IgG HRP conjugated antibodies addition and incubation

- **Wash step** → Dispense and aspirate 200 µl of 1X wash buffer five times in all wells.
- After washing, thoroughly dispose of all liquid from the microplate by tapping it on an absorbent paper with the openings facing downwards to remove all residual wash buffer.
- In each well, pipette 100 µl of anti-human **IgM** conjugated with HRP in rows A, B, C, and D.
- In each well, pipette 100 µl of anti-human **IgG** conjugated with HRP in rows E, F, G, and H.
- Cover the plate and incubate for 1 hour at room temperature.

iii. Enzyme substrate solution addition and incubation

- **Wash step** → Dispense and aspirate 200 µl of 1X wash buffer five times in all wells.
- After washing, thoroughly dispose of all liquid from the microplate by tapping it on an absorbent paper with the openings facing downwards to remove all residual wash buffer.

IMPORTANT: Enzyme substrate solution (TMB) must reach room temperature before use. The enzyme substrate solution must be clear to use. **DO NOT USE if the solution is colored.**

- Add **100 µl** of enzyme substrate solution (TMB) in all wells.
- Cover the plate and incubate in the dark (example; covering with aluminum foil) at room temperature.
- Incubate plate for a total of **30 min** at room temperature.

iv. Stop solution addition and plate reading at 450 nm

- Add **100 µl** of stop solution (H_2SO_4) in all well.
- Read the optical absorbance immediately at 450 nm (bottom reading).
- Resulting optical density (OD) values will be used to calculate patient (number 1 to 20) responses to TICKPLEX® BASIC antigens.

8. CALCULATION OF RESULTS

For manual calculations, the quality control certificate provided with the test kit and the plate layout provided above must be followed along with the instructions in this section.

i. Optical density index (ODI) calculations

- **Evaluate validity of the test performed for ODI analysis**

  The test performed is valid if;

  1. The OD values for IgM (well A1) and IgG (well E1) **positive controls are ≥ 1.0**
  2. The OD values for IgM (well B2) and IgG (well F2) **negative controls are ≤ 0.5**

- **Calculate individual cut-off values for antigens A1 and A2**

  1. **For IgM test result** → Insert optical density values from wells C1 and D1 for IgM calibrator control in Table 2 provided in the quality control certificate (last row named “Tested results reads 5 and 6”).
  2. **For IgG test result** → Insert optical density values from wells G1 and H1 for IgG calibrator control in Table 3 provided in the quality control certificate (last row named “Tested results reads 5 and 6”).
  3. Calculate the mean for IgM and IgG calibrators in Tables 2 and 3 of the quality control certificate.
  4. Multiply each IgM and IgG mean with the correction factor in Table 4 of the quality control certificate to obtain cut-off values for antigens A1 and A2.

- **Calculate the ODI**

  1. For each antigen (A1 and A2) and patient (patient 1 to 20), divide the OD value over its cut-off value calculated above.

For IgM and IgG, calculate the ODI according to the following formula,

$$\frac{\textbf{OD value of a patient for antigen A\#}}{\textbf{Cut off value of antigen A\#}} = \textbf{ODI}$$

where, A# = Antigen A1 or A2

Interpret results as follows,

ODI < 0.9	**NEGATIVE**
ODI ≥ 0.9 to < 1.0	**BORDERLINE**
ODI ≥ 1	**POSITIVE**

ii. Antibody titer calculations

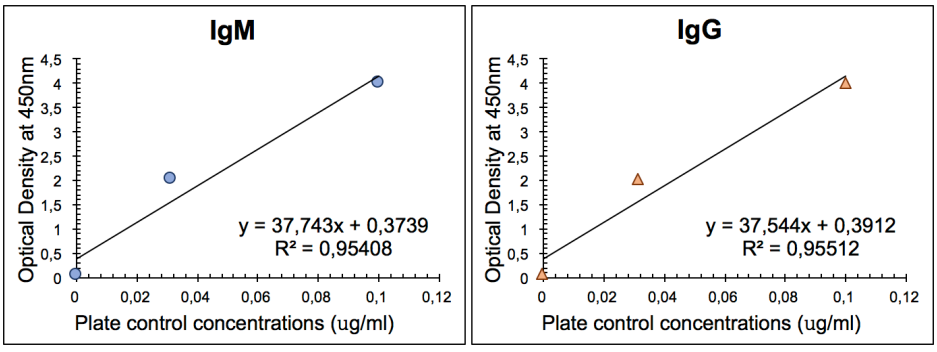
- **Create a standard curve for IgM and IgG**

1. Use a computer to create a scatter plot with OD-values of negative control, calibrator 1, and positive control on the y-axis and their corresponding concentrations on the x-axis in the same order as presented below in Table 2.

Table 2. Negative control, Calibrator, and Positive control concentrations (in *µ*g/ml) for the standard curve.

Controls required for creating a standard curve	Reference well number		Concentration (*µ*g/ml)	
	IgM	IgG	IgM	IgG
Negative control	B1	F1	0.000	0.000
Calibrator 1	C1 and D1	G1 and H1	0.003	0.006
Positive control	A1	E1	0.100	0.100

2. Add a linear trend line with a straight-line equation, and R-squared (R^2) value to the scatter plot. The following plots are an example of a typical standard curve. DO NOT use the standard curves below to determine antibody concentrations in patient samples.



- **Evaluate validity of the test performed for antibody titer analysis**

The test is valid if,

1. The **R^2 value is ≥ 0.75** for IgM and IgG.

- **Calculate antibody concentrations in patient samples**

  Use the straight-line equation ($y = mx \pm c$) from IgM and IgG standard curves.

  1. In place of "y", insert OD value of an antigen for a patient and solve for "x". Resulting value for "x" is the antibody concentration in µg/ml.

iii. Test result interpretation

It is recommended that both ODI and corresponding antibody titer values are taken into clinical consideration. A negative immune response on TICKPLEX® BASIC does not preclude the possibility of a positive immune response to microbes associated with tick-borne disease: false negative immune responses can be due to stage of infection (e.g., specimen obtained prior to the development of cellular immune response), co-morbid conditions which affect immune functions, incorrect handling of the blood collection tubes following venipuncture, incorrect performance of the assay, or other immunological variables. Patients in early stages of infection may not produce detectable levels of antibody. Further, early antibiotic therapy after ECM (*erythema chronicum migrans*) may diminish or abrogate good antibody response. Immunocompromised patients may never generate detectable antibody levels. A positive immune response on TICKPLEX® BASIC result should not be the sole or definitive basis for determining infection with tick-borne pathogen. Incorrect performance of the assay may cause false-positive responses. A positive immune response on TICKPLEX® BASIC should be followed by further medical evaluation and diagnostic evaluation for an active tick-borne disease. Sera/plasma from patients with other spirochetal diseases (syphilis, yaws, pinta, leptospirosis and relapsing fever), infectious mononucleosis or systemic lupus erythematosus may give false positive results.
The IgM antibody test can be used for asymptomatic patients a minimum of 2 weeks after confirmed tick bite.
The IgG antibody test can be used for asymptomatic patients a minimum of 4 weeks after confirmed tick bite.
The IgM antibody test can be used for tick-borne diseases symptomatic patients a minimum of 2 weeks after confirmed tick bite.
The IgG antibody test can be used for tick-borne diseases symptomatic patients a minimum of 4 weeks after confirmed tick bite.

9. CHARACTERISTICS OF THE TEST

i. Validity of the test

The test is intended for determination of specific IgM and IgG antibody responses in human serum or plasma. Te?ted Oy does not guarantee kit operation with EDTA plasma, heparin plasma, citrate plasma, or hemolytic serum/plasma. The test can be performed on minimum 4 ml of whole drawn blood, on samples stored generally at +4°C for up to 14 days, on diluted samples within one working day, and on samples stored at -20°C for up to one year.

ii. Precision of test and coefficient of variation

Coefficient of variation was assessed by calculating intra- and inter-assay variation. Intra-assay variation was determined by a duplicate high titer, and low titer measurement from the same plate. For inter-assay, variation was determined by measuring three high titer samples and three low titer samples from different plates that were performed on three different days. Coefficient of intra-assay variation is max 4.25%, and the coefficient of inter-assay variation is max 4.29%.

iii. Clinical studies

Please refer to the peer-reviewed article Garg, K et al. *Trop. Med. Infect. Dis.* **2021**, *6*, 38.

iv. Diagnostic sensitivity and specificity

Table 2. The diagnostic sensitivity, specificity, and their evaluation has been tabulated.

Antigen	IgM/IgG Sensitivity	IgM/IgG Specificity	Evaluation criteria
Borrelia species and persistent Borrelia forms	95%	98%	Previous clinical diagnostic results for 331 patients with Lyme disease were compared with test results from the Tickplex study performed at the JYU University.

10. SAFETY PRECAUTIONS

- For *in vitro* diagnostic use only.
-When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. Do not smoke, drink, or eat while performing or preparing for the assay. Do not pipette by mouth. Wear disposable gloves while handling reagents or samples and wash your hands thoroughly afterwards. Avoid spilling or producing aerosols.
-Do not use kit if any reagent bottle or the microplate shows signs of damage or leakage prior to use. Do not use blood collection tubes or ELISA kit after the expiration date.
-Ensure that laboratory equipment such as plate washers and readers have been calibrated / validated before use.
-Handle stop solution (H_2SO_4) with care. Avoid contact with skin or mucous membranes. In case of contact with skin, rinse immediately with plenty of water and seek for medical advice. Liquid waste containing acid [stop solution (H_2SO_4)] should be neutralized in 4% sodium bicarbonate solution.
-Handle human blood as if potentially infectious. Observe relevant blood handling guidelines. While preparing and perform the assay, all material that encountered human serum should be considered as contagious and therefore should be handled / disposed according to appropriate regulations.
- If not using the complete solutions at one time, use in sterile conditions to prevent contamination.

11. HANDLING PRECAUTIONS

-Follow assay procedure indicated above.
-Avoid microbial contamination of serum samples and kit reagents.
-If not using the complete solutions at one time, use sterile conditions to prevent contamination.
-Avoid cross-contamination of reagents.
-Avoid contact of enzyme substrate solution (TMB) with oxidizing agents, metal surfaces, and light.
-Enzyme substrate solution (TMB) must be clear on use; do not use if the solution is colored.
-Store reagents in conditions mentioned above when not in use.
-Do not re-use diluted human antibodies or IgM/IgG conjugated with HRP samples in 1% sample buffer. Always prepare fresh.
-Do not use reagents after the expiry date printed on the label.
-Variations in the test results are usually due to:

- Insufficient mixing of reagents and samples
- Inaccurate pipetting and inadequate incubation times

- Poor washing techniques or spilling the rim of well with sample or IgM/IgG conjugated with HRP
- Use of identical pipette tips for different solutions
- The ELISA microplate and assay reagents such as enzyme substrate solution (TMB) did not reach room temperature +15°C to +25°C

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Technical Assistance: info@tezted.com

More information: www.tezted.com

UDI-DI: 111745457308 (Tickplex Basic)