REVIEW ARTICLE

# A review of ten years of *Leptospira* serology and PCR testing

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## ABSTRACT

**Objective:** To evaluate the use of diagnostic *Leptospira* laboratory testing within the Midlands region of New Zealand and identify the most sensitive testing strategy.

**Method:** *Leptospira* serology and polymerase chain reaction (PCR) testing performed within the Pathlab remit in 2013-2022 were reviewed by comparing methodologies and request origins to identify trends over time and evaluate the relative performance of serology and PCR testing.

**Results:** 81% of the cases tested by both serology and PCR that were confirmed infections were detected by either blood PCR, urine PCR, or serology alone. No significant difference was observed between the detection rates of blood PCR and urine PCR. Serology was the most frequently requested methodology, though PCR testing quadrupled in 2017 and uptake has continued to increase since then, becoming the favoured methodology amongst hospital-based requestors in 2022. Appropriately timed paired serological testing was rarely performed.

**Conclusions:** No single methodology can be relied on to consistently detect leptospirosis infections. Follow-up serology was under-utilised. A combination of PCR and serology testing was the most effective testing strategy.

**Key words:** Leptospirosis, ELISA, microscopic agglutination test (MAT), serology, PCR.

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## INTRODUCTION

Leptospirosis is an illness caused by infection with pathogenic spirochaetes of the *Leptospira* genus and is a notifiable disease in New Zealand, which has an annual incidence of approximately 2 per 100,000 population (1). Transmission is typically zoonotic, occurring through contact with infected farm animals or rats, or through contact with water or soil contaminated with the urine of infected animals. Risk of exposure is therefore highest for rural occupations and meat workers (2).

After a mean incubation period of ten days, leptospirosis causes a biphasic illness with a range of presentations. During the acute phase, leptospires are present throughout the blood and tissues, commonly causing flu-like symptoms such as headache, fever, myalgia and conjunctival suffusion. In the 10% of cases that are severe, Weil's disease (jaundice, renal failure, haemoptysis, and dyspnoea), meningitis, or respiratory failure can develop. After three to nine days, the acute phase may cede to a brief asymptomatic period before the immune phase begins. The immune phase is characterised by a rise in anti-*Leptospira* IgM antibodies and clearance of leptospires from the blood and tissues, except kidney tubules, resulting in prolonged intermittent shedding in the urine. This phase can present similarly to the acute-phase symptoms, which may progress to multiple organ failure (3,4). When clinical suspicion for leptospirosis is high, treatment with antibiotics is initiated immediately, as treatment is most effective when commenced within five days of illness (4).

The non-specific and variable manifestations of leptospirosis make the infection difficult to identify by clinical presentation alone. The presentation may be similar to other conditions such as viral hepatitis, influenza, toxoplasmosis, and septicaemia; other rural infections, such as rickettsiosis; or tropical diseases in the case of returned travellers (3,4). This makes laboratory findings essential to confirming the diagnosis. Leptospirosis tests available in New Zealand include serology and polymerase chain reaction (PCR). Two types of serological tests are used for leptospirosis diagnosis in New Zealand: *Leptospira* IgM by enzyme-linked immunosorbent assay (ELISA) or chemiluminescent immunoassay (CLIA), and the microscopic agglutination test (MAT). The IgM assays qualitatively detect the presence of anti-*Leptospira* IgM antibodies and are commonly employed as a screening test with the MAT used as a confirmatory assay. *Leptospira* IgM assays can be performed on automated platforms with a short turn-around-time. However, a positive *Leptospira* IgM result is not confirmatory for leptospirosis as it may represent a recent infection, a cross-reaction, or a past infection, since anti-*Leptospira* antibodies can remain detectable for months to years post-infection (3,5). MAT is regarded as the gold standard serological test for leptospirosis (2,3), though it also has drawbacks. The method involves the use of a panel of live *Leptospira* serovars. Antibodies of both IgG and IgM isotypes from patient serum bind to the causative serovar in the panel, resulting in agglutination and therefore identification of the infecting serovar (6). A minimum of 50% agglutination at a titre ≥ 400 in a single serum sample, or a minimum four-fold rise between acute and convalescent sera titres is considered serological confirmation of leptospirosis in New Zealand (2). The two diagnostic laboratories where *Leptospira* MAT is available in New Zealand use the same panel of eight serovars known to cause infection in New Zealand and Australia. As there are over 250 pathogenic *Leptospira* serovars worldwide, leptospirosis acquired overseas may give false negative results if the infecting serovar is not present in the MAT panel (2,6). The restriction of MAT to only two laboratories nationwide is a result of its complex and labour-intensive nature. The need to maintain live *Leptospira* cultures presents both a technical difficulty and a biosafety hazard. The test cannot be standardised and must be maintained as an in-house assay and has an interpretation that may be subject to reader variation (6,7).

The sensitivity of serological tests for leptospirosis varies with the stage of infection. Testing should therefore be carried out on temporally paired sera. Both *Leptospira* IgM and MAT have low sensitivity during the acute phase of infection, when the humoral immune response is yet to appear. Sensitivity becomes optimal during the immune phase (8). In practice, this means that a convalescent sample of an infected patient, which is recommended to be taken three weeks after the onset of symptoms (4), compared to the acute sample taken at the first presentation, should show either seroconversion or a rise in MAT titre (7,9), providing a retrospective diagnosis. In some cases, early antibiotic therapy can interfere with the antibody response, and repeat testing beyond the paired sera may be necessary (4,7).

In contrast to serology, PCR can provide a more rapid "real-time" laboratory diagnosis of leptospirosis using blood or urine samples, and in cases of meningitis, cerebrospinal fluid (CSF) (4). PCR on blood samples can detect leptospirosis during the first week of symptomatic illness, before antibodies are detectable by serological methods (10,11). However, leptospiraemia is efficiently cleared during the immune phase, resulting in blood PCR becoming unreliable from the second week of illness (11). Urine is the recommended PCR sample-type during the immune phase. However, as leptospires are shed intermittently from the kidneys during infection, a negative urine PCR result does not exclude leptospirosis and should be repeated in cases with high clinical suspicion of leptospirosis (2,4). A single positive PCR result is sufficient for confirmation of leptospirosis (2).

Testing for leptospirosis is inconsistent throughout New Zealand, as test accessibility, which is dependent on location, dictates how

a patient is tested, as opposed to official guidance. *Leptospira* IgM is performed by three diagnostic laboratories using different test kits and platforms. All positive and equivocal *Leptospira* IgM samples are sent to the same reference laboratory for MAT. Patients who have *Leptospira* serology performed outside the areas covered by these laboratories are only tested by MAT, not IgM ELISA or CLIA, and only when both acute and convalescent serum samples have been submitted (with exceptions). PCR testing is also available at three laboratories, and acceptance criteria for testing varies from none to provision of specific clinical details and sample timing (unpublished survey of New Zealand's medical laboratories, November 2021). Guidance for laboratory testing is also inconsistent. The Ministry of Health recommends that patients be tested by both MAT and PCR (2). This conflicts with advice from Best Practice Advocacy Centre New Zealand (bpac$^{nz}$) to perform paired serology and only add PCR when illness is severe or if it is necessary to confirm an occupationally acquired infection (4).

This study undertook a retrospective review of *Leptospira* serology and PCR results over a ten-year period from patients in the Pathlab remit, to evaluate the utilisation and value of the different test methods available for diagnosing leptospirosis in the Midlands region. The aim was to identify which testing strategies have been most effective in detecting leptospirosis.

## METHODS

### Ethics

Ethical approval was not required as per the Health and Disability Ethics Committees' (NZ) screening questionnaire.

### Data Collection

*Leptospira* serology and PCR results were retrieved from the Pathlab results repository accompanied by patient National Health Index (NHI), requestor location, sample identifier, and collection date, to cover 2013–2022, inclusive. The retrieval captured requests from community-based requestors throughout the Midlands region of New Zealand, excluding Tairāwhiti and Taranaki, in addition to four public hospitals located in Tauranga, Whakatāne, Rotorua, and Taupō. Requests collected at Waikato, Thames, Tokoroa, Te Kuiti, and Taumarunui Hospitals do not fall under the Pathlab testing remit and were not included in the data.

Serology consisted of IgM ELISA (PanBio) performed at Pathlab Waikato, and MAT performed by ESR Wallaceville on samples equivocal or positive by IgM ELISA. PCR was performed at Waikato Hospital Laboratory, initially almost exclusively on blood samples, with urine officially validated as a sample-type in 2017.

### Result Interpretation

IgM ELISA results were reported qualitatively as either negative, equivocal, or positive, as per manufacturer cut-offs. MAT results were reported quantitatively as a titre, as well as with a qualitative interpretation. Interpretations included "negative" (titre < 50), "exposed" (titre 50–200, where only one sample was collected, or the titres from paired sera remained within this range without a four-fold rise), and "confirmed" (single titre ≥ 400, or four-fold increase in titre between acute and convalescent sera). PCR results were reported qualitatively as "not detected" or "detected". Any case with a confirmed MAT result and/or PCR detected was regarded as confirmed positive for leptospirosis, as per the Ministry of Health New Zealand's laboratory criteria for diagnosis (2).

### Data Analysis

Serology and PCR test numbers, and PCR sample-types used, were compared for hospital- and community-based requestors over time. The relative performance of serology and PCR tests was analysed by applying an inclusion criterion of both serology and PCR results being available for each case of suspected leptospirosis. Test results grouped for each case were required to pertain to the same episode of illness. Where multiple requests were made for the same test during the same episode of illness and the result changed between requests, the convalescent or confirmatory results were deemed to be most useful and used for the purposes of data analysis.

Using the data that met the inclusion criterion, the negative predictive value (NPV) of *Leptospira* IgM ELISA was calculated as the number of cases where IgM and PCR were negative (IgM true-negative) divided by the total number of cases where IgM was negative. The positive predictive value (PPV) of *Leptospira* IgM ELISA was calculated as the number of cases where IgM was positive and supported by a confirmed MAT or detection by PCR (IgM true-positive) divided by the total number of cases where IgM was positive. Equivocal IgM results were excluded from these calculations as they defer to the MAT result.

Results preceding 2017 were removed to give an overview of how clinicians requested *Leptospira*-specific tests when serology, blood PCR, and urine PCR were all available as options, and to review the outcomes of the different testing strategies. A further inclusion criterion of both blood and urine PCR results being available was applied to compare the relative performance of sample-types using McNemar's test (12). Finally, a subset of requests from 2022 was analysed to gauge the frequency at which serology is followed up with convalescent sample testing.

**Table 1.** Summary of results for all cases that had both *Leptospira* serology and PCR tests performed.

| | | Serology | | | | | |
|---|---|---|---|---|---|---|---|
| | | IgM N | IgM Eq, MAT N | IgM Eq, MAT Ex | IgM P, MAT N | IgM P, MAT Ex | IgM P, MAT CONF |
| PCR | Bl & Ur ND | 86 | 0 | 1 | 8 | 4 | **5** |
| | Bl ND, Ur NT | 56 | 1 | 1 | 2 | 1 | **3** |
| | Bl NT, Ur ND | 36 | 1 | 0 | 0 | 1 | **5** |
| | Bl & Ur DET | **1** | 0 | 0 | 0 | 0 | **1** |
| | Bl DET, Ur ND | **4** | 0 | 0 | 0 | **1** | 0 |
| | Bl DET, Ur NT | **3** | **1** | 0 | 0 | 0 | **1** |
| | Bl ND, Ur DET | **9** | 0 | 0 | 0 | 0 | **5** |
| | Bl NT, Ur DET | **2** | **1** | 0 | 0 | **1** | 0 |

N = negative; Eq = equivocal; Ex = exposed; P = positive; CONF = confirmed; Bl = blood; Ur = urine; ND = not detected; NT = not tested; DET = detected. Numbers in **bold** are cases meeting the laboratory criteria for confirmation of leptospirosis.

**Table 2.** Testing modalities requested for suspected cases of leptospirosis in 2017-2022.

| | Ser & PCR | Ser only | Bl & Ur PCR only | Bl PCR only | Ur PCR only |
|---|---|---|---|---|---|
| **Total cases** | 207 | 1,353 | 151 | 49 | 32 |
| **Unconfirmed cases** | 173 | 1,308 | 136 | 49 | 31 |
| **Confirmed cases (%)** | 34 (16%) | 45 (3%) | 15 (10%) | 0 (0%) | 1 (3%) |

Ser = serology; Bl = blood; Ur = urine

**Table 3.** PCR results for each sample-type belonging to cases who had serology and both PCR sample-types tested.

| | | Blood | | Total |
|---|---|---|---|---|
| | | Detected | Not Detected | |
| **Urine** | Detected | 2 | 14 | 16 |
| | Not detected | 5 | 104 | 109 |
| **Total** | | 7 | 118 | 125 |

## RESULTS

The data collected for the study period included 3,703 *Leptospira* IgM and 843 PCR tests from 3,344 patients. As CSF PCR was performed on only three patients, data on CSF was excluded from this review.

The results of serology and PCR testing are summarised in Table 1 using only the cases that met the inclusion criterion of having both serology and PCR results (241 cases). 43 cases (18%) met the laboratory case-definition for confirmed leptospirosis. Of those 43 cases, 23 (53%) met the laboratory case-definition for leptospirosis due to PCR testing only. 35 (81%) confirmed cases were detected by only one of the three possible confirmatory tests: Blood PCR alone was responsible for 9 (21%) confirmed cases, urine PCR for 13 (30%), and MAT for 13 (30%). IgM ELISA had a NPV of 90% compared to PCR, and a PPV of 58% compared to MAT and PCR.

The number of cases tested by each available combination of testing methodologies during the years 2017-2022 and the proportion that returned confirmatory results is summarised in Table 2.

The 125 cases with both blood and urine PCR results are summarised in Table 3, from which McNemar's test two-tail p-value was calculated to be 0.06. Using a significance threshold of 0.05, this provides insufficient evidence for a difference in the proportion of detected results between blood and urine PCR sample-types.

## DISCUSSION

In the community setting, *Leptospira* IgM testing decreased throughout the study period (Figure 1). Uptake of PCR in the community during this period never reached sufficient volume to explain, by way of replacement, the continual and marked decline in IgM testing illustrated in Figure 1. Conversely, serology requests originating from hospitals remained steady throughout, even as PCR was adopted, with PCR eventually becoming the more frequently used methodology for this requestor group in 2022 (Figure 2). A survey to investigate the cause of these trends is out of scope for the current study, but we suggest that the availability of *Leptospira* PCR, a test performed at Waikato Hospital Laboratory, was better known to hospital-based requestors, and GPs may have been unaware that they had access to PCR testing.

As illustrated in Figures 2-4, an increase in the number of *Leptospira* PCR requests and confirmed cases was seen in 2017. This year's quadrupling in total PCR requests coincided with the year urine became a validated PCR sample-type at Waikato Hospital, though the increased requests were for both sample types (Figure 4). 2017 also saw a peak in the national notification rate of leptospirosis at 3 cases per 100,000 compared to 1.8 cases per 100,000 the previous year. Additionally, in this peak year, Waikato had the highest notification rate for leptospirosis, nationwide, at 13.2 cases per 100,000 (13). Beyond the new availability of urine as a PCR sample-type raising awareness of *Leptospira* testing, reasons for the 2017 spike in test numbers and confirmations are unclear.

Since the upsurge in 2017, it can be seen in Figure 2 that the proportion of *Leptospira* test requests that were for PCR increased with a concurrent upwards trend in the number of confirmed cases. The two spikes in confirmed cases in 2017 and 2021 (Figures 2 and 3) are matched by spikes in community PCR testing (Figure 4), and a relatively small spike in community IgM testing for 2021 only but are not reflected in the proportion of non-negative IgM results, which is steady in 2017 and at a trough in 2021 (Figure 5). This indicates that PCR detected cases that were missed or not tested by IgM ELISA during these spikes in testing.

We have reported a low PPV of 58% for IgM ELISA, which does not correlate with the performance reported by other studies (8,9,14). This could be attributed to calculating the PPV based on the corresponding MAT and/or PCR result for each positive IgM ELISA. Research has suggested that the non-serovar-specific IgM detected by ELISA can be detected earlier than the serovar-specific antibodies detected by MAT (6,15). Therefore, the non-confirmed MAT results, taken from a population that has been demonstrated not to test serology routinely with paired sera (Figure 6), may have falsely lowered the IgM ELISA's PPV. Eugene et al. calculated a PPV of 80% for IgM ELISA by using Bayesian latent class modelling to account for the unreliability of MAT in the acute phase (9), which supports the notion that the performance of IgM ELISA may be better than our data suggests.

Analysis of the relative performances of the differing methodologies showed serological testing alone to be responsible for confirming leptospirosis in 30% of cases where both serology and PCR had been performed. This is consistent with Earl et al.'s finding that 36% of the leptospirosis-positive patients enrolled in their study did not have their illness confirmed by PCR (5). In our data review, when both serology and PCR were tested, a greater proportion of the confirmed cases (53%, 23/43) were detected by PCR only. It is not surprising that only 17% (2/12) of the cases that tested positive for blood PCR were also confirmed by serology compared to the 32% (6/19) of urine cases that were also confirmed by serology. This is because the acute leptospiraemic phase is the only window when blood PCR can detect an infection, and this window precedes the antibody response. Cases that are confirmed by blood PCR in the acute phase do not require follow-up serology unless it is deemed necessary to identify the infecting serovar for Public Health purposes. Leptospires are cleared from the blood and intermittently shed in the urine at the time when the antibody response becomes detectable (4), thereby explaining the higher incidence of urine PCR and serology co-confirmations. While urine PCR was responsible for confirming more cases than blood PCR, a comparison of the two sample-types by McNemar's test did not indicate that one detected leptospirosis significantly more than the other. This, in addition to the fact that 81% (35/43) of confirmed cases were detected by only one of the three possible confirmatory tests, indicates that the combined use of serology, blood PCR, and urine PCR is the most sensitive strategy for detecting leptospirosis in suspected cases. This is evident in Table 2, where combined serology and PCR testing gave the highest proportion of cases detected. Earl et al.'s study came to the same conclusion that blood and urine PCR and serology should all be employed for leptospirosis laboratory investigations (5). Additionally, a review by Budihal and Perwez of various laboratory tests for leptospirosis concluded that PCR and IgM ELISA used together is the most effective way to achieve an early diagnosis of leptospirosis (16).

We recommend that New Zealand incorporate this multi-modal approach into the development of a national standardised testing strategy for leptospirosis that optimises case detection, by way of judicious test selection, in an equitable fashion. In order to implement this, further work is required, such as evaluating the worth of the additional expense incurred by increased testing by

a cost-benefit analysis.

While this review has shown that testing by both serology and PCR has been the most sensitive approach for detecting leptospirosis, the sensitivity of either methodology can be undermined by inappropriate sample collection or sample-type. Blood PCR is not indicated after the first week of illness, but appropriate sample timing could not be assessed for our data as date of symptom onset was not available. The timing of serum collection is also important. Earl et al. reported that 84% of patients with suspected leptospirosis presented to their general practitioner during the acute phase of the illness (5). At this time, the antibody response is usually undetectable and a negative *Leptospira* IgM result is to be expected, which should be followed up with a convalescent sample. The importance of testing convalescent samples was demonstrated by Bajani et al., who calculated the sensitivity of IgM ELISA to be 49% in acute sera and 75% in convalescent sera, with MAT also being 49% in acute sera but rising to 94% in convalescent sera (8).

Our analysis of serology testing from 2022 (Figure 6) suggests that sample timing is not performed optimally. That year, no follow-up serology was collected for 87% of cases where serology was performed, and leptospirosis was not confirmed by initial serology or PCR (Figure 6). Of the 5.9% that had the follow-up serum collected, more than half had it collected earlier than recommended. This is despite each initial negative *Leptospira* IgM result being reported with a comment recommending repeat serology in 3-4 weeks. This rate is comparable to that seen by Waikato Hospital Laboratory, where in 2013, only 16% of *Leptospira* serology requests were followed up with a convalescent sample (17). Earl et al. found that, even with prompting from their medical centre, 32% (15/47) of patients in their study did not return for follow-up serology, and the researchers estimated that up to 31% (4/13) of patients who did not return could have had leptospirosis which was not detected by acute-phase tests (5). It is not always necessary to test follow-up serology, such as when the diagnosis of a different illness is made; also, patients may move between different laboratory remits, resulting in their acute and convalescent samples being tested by different laboratories, giving the false appearance of follow-up serology not being performed. However, these factors are unlikely to fully explain the relative frequency of 2.7% for appropriately paired IgM ELISA samples reported here. In light of this suboptimal use of *Leptospira* IgM ELISA, investigation into its value compared to other methods used outside New Zealand, such as point of care testing, may be of interest.

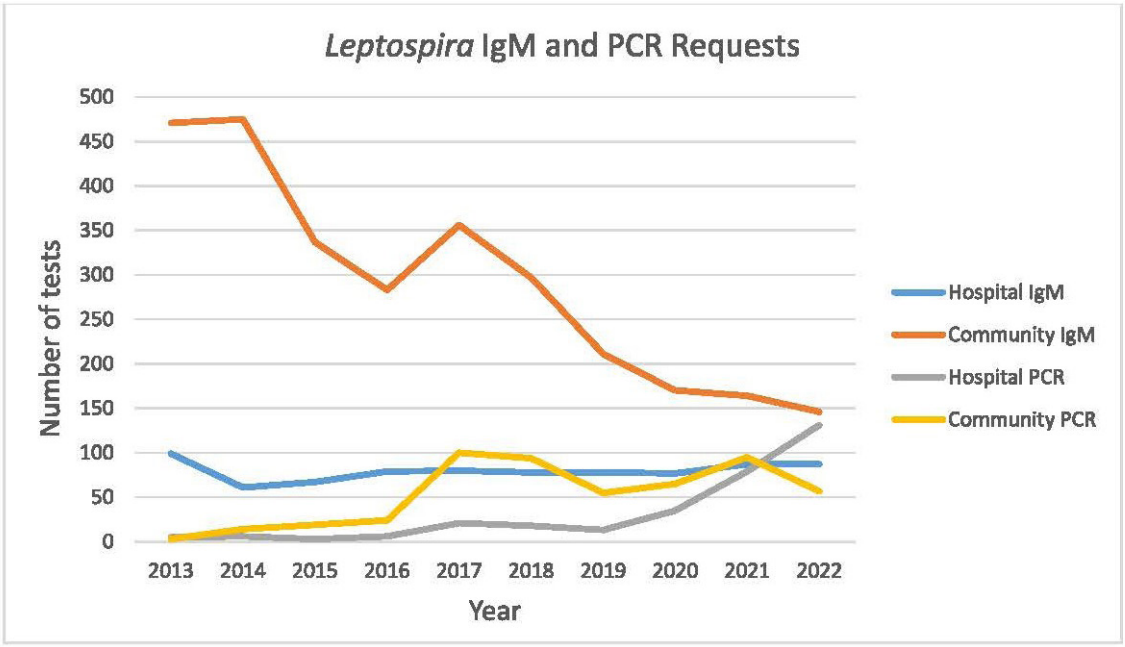


**Figure 1.** Uptake of Leptospira IgM and PCR testing by community- and hospital-based requestors over time.

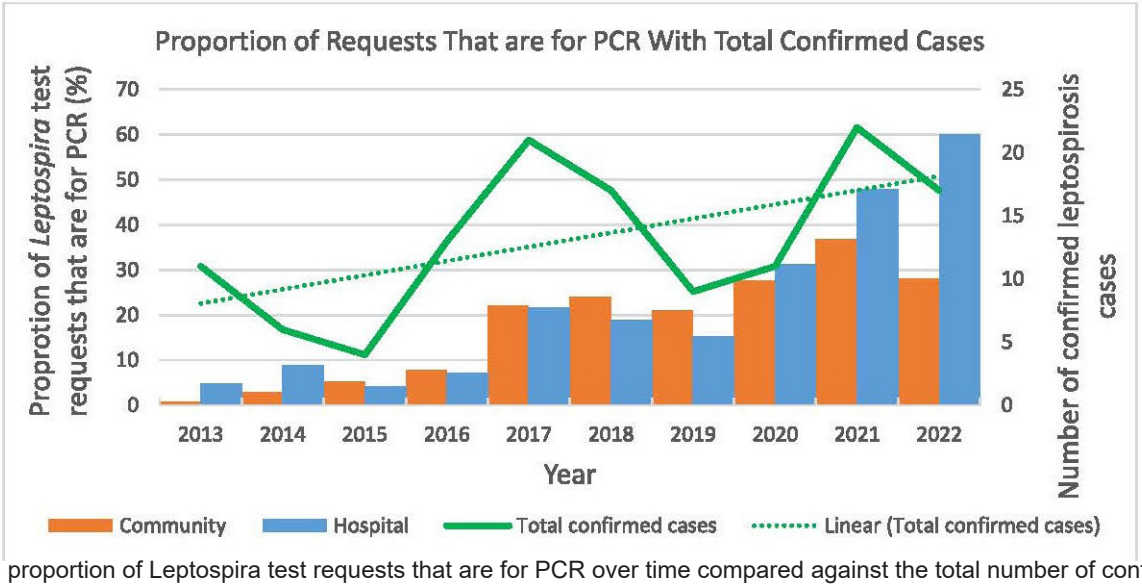


**Figure 2**. The proportion of Leptospira test requests that are for PCR over time compared against the total number of confirmed leptospirosis cases in the data set, with the linear relationship between the total annual number of confirmed cases and time demonstrated (r = 0.56 by Pearson Correlation).

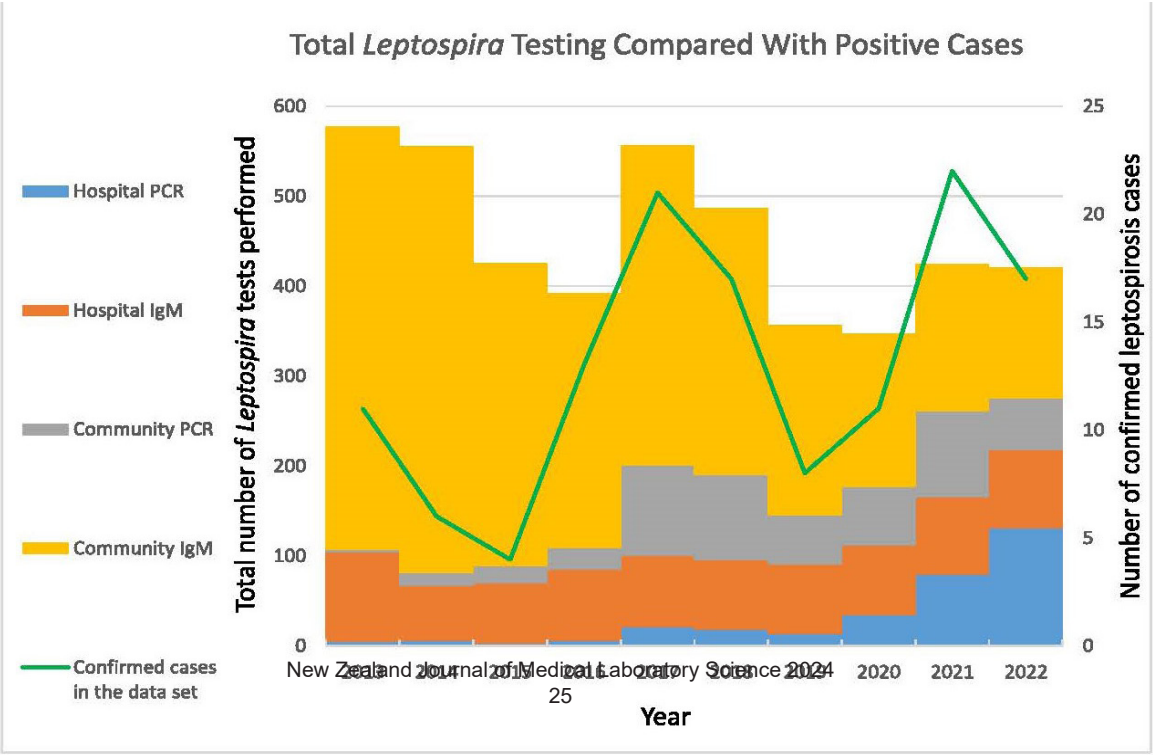


**Figure 3.** Overall test numbers grouped by requestor and test type over time, with our data set's confirmed case numbers overlaid.

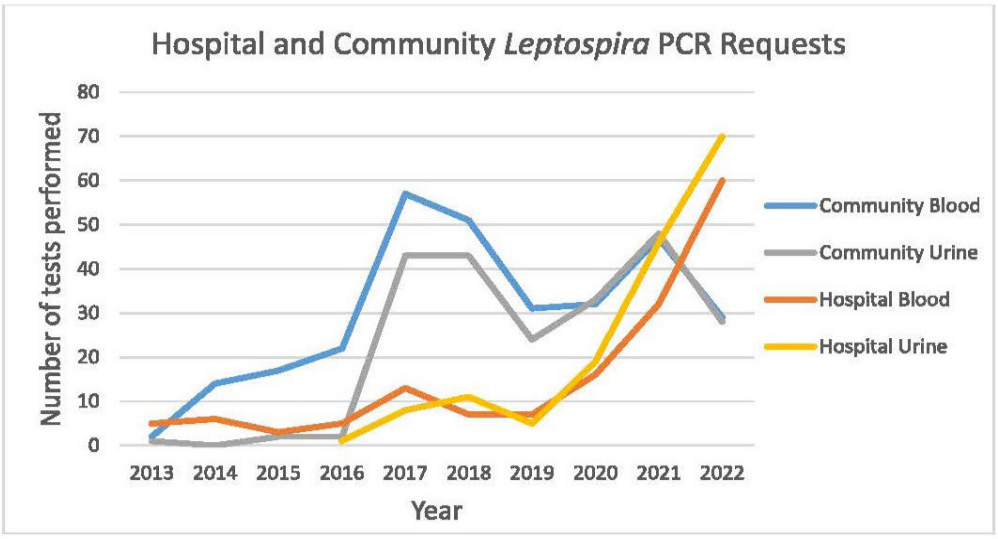


**Figure 4.** Number of blood and urine Leptospira PCR tests per year, originating from community- and hospital-based requestors.

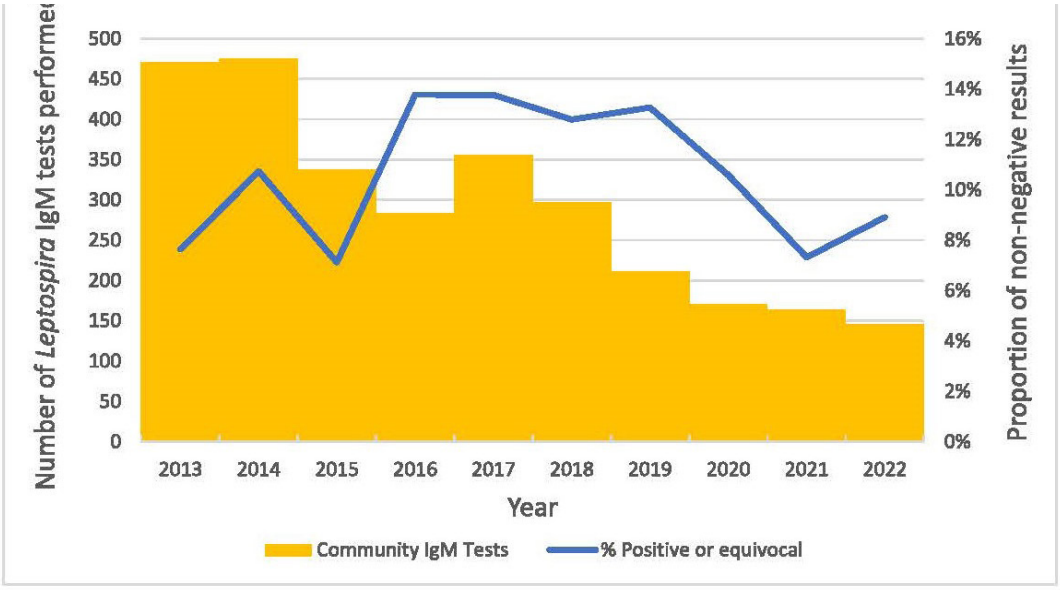


**Figure 5.** The total number of community-based Leptospira IgM tests over time, with the proportion of those that gave a non-negative (equivocal or positive) result overlaid.

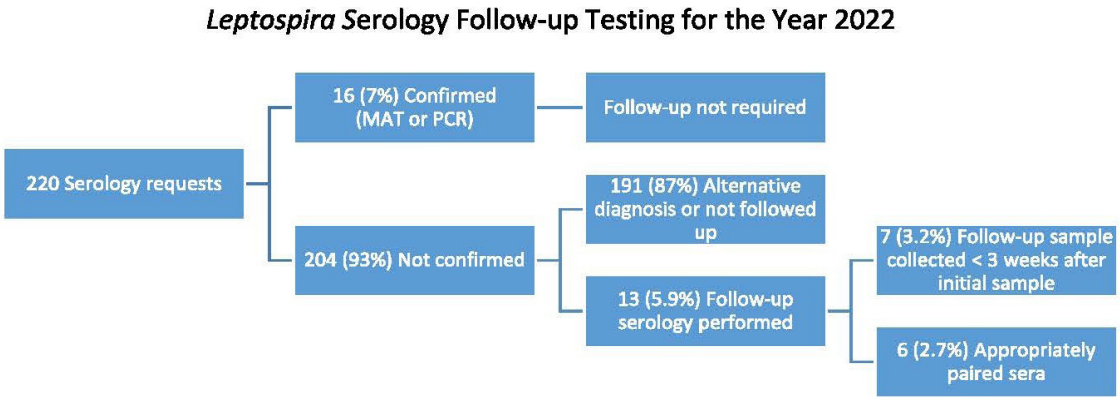


**Figure 6:** Leptospira serology requests for the year 2022, and the cases followed-up within appropriate and inappropriate timeframes.

**Limitations**

As leptospirosis is a biphasic illness, studies into *Leptospira*-specific tests often differentiate acute and convalescent results. Such differentiation was not possible here as this information is not a pre-requisite for testing and is seldom provided to the laboratory. Where the identification of follow-up testing was necessary, a presumptive status was assigned primarily by correlating sample collection dates in addition to manual case-by-case review of clinical details provided to the laboratory. The lack of information provided on sample timing relative to symptom onset also precluded any analysis into the appropriateness of blood PCR sample timing.

## CONCLUSION

Laboratory diagnosis of leptospirosis in New Zealand remains both challenging and geographically heterogeneous. Our data indicated that follow-up serology is under-utilised. A combination of PCR and serology has been shown to be the most effective testing strategy, as no one test modality captures all clinical cases.


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