PREVALENCE AND DIAGNOSIS OF THE

ETIOLOGIC AGENTS OF PYREXIA OF

UNKNOWN ORIGIN IN THE

CENTRAL QUEENSLAND REGION

by

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A thesis submitted for the degree of

Master of Applied Science

University of Central Queensland

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May 1994

ABSTRACT

A study was undertaken to ascertain the prevalence of agents which may be associated with pyrexia in the Central Queensland region. Five hundred and twenty four patient sera submitted to the laboratory from January 1991 to December 1992 for Ross River virus serology were also assayed by serological methods for evidence of exposure to Barmah Forest virus, flaviviruses, spotted fever group rickettsia, brucella, leptospirosis and lyme borrelosis. Titres of streptococcal antibodies and rheumatoid factor were also measured. Accompanying request forms were analysed for relevant clinical history and any additional pathology testing requests were noted.

Ross River virus antibodies (IgG and/or IgM) were found in 38.9% of patient sera. Males were significantly associated with the presence of antibodies (IgG and/or IgM) ($P < 0.05$) but females were 1.73 times as likely to have detectable IgM antibody at presentation. This suggests that females may be more symptomatic in the initial stages of infection and thus seek medical intervention. Flavivirus antibodies (IgG) were found in 19.7% of patient sera. One sample also contained IgM antibodies which reacted with both Kunjin and Alfuy viruses. Barmah Forest virus antibodies (IgG and/or IgM) were detected in 8% of patient sera. IgM antibody was detected in three patients, two of whom also had Ross River virus IgM antibody. Antibodies (IgG and/or IgM) to the spotted fever group of rickettsia were detected in 3.8% of patients. Lyme borrelosis antibodies (IgG) were detected in 1.7% of patients. A single case of brucellosis was diagnosed but no evidence of exposure to leptospiras was found. Raised streptococcal antibody titres were found in 27.1% of patient sera. Rheumatoid factor was detected in 4.6% of patient sera. A full blood count, erythrocyte sedimentation rate and rheumatoid factor determination were the most commonly requested additional pathology tests. Clinical history was provided on only 56.1% of request forms.

These results have implications for future testing protocols. Diagnostic problems associated with serological testing on single serum samples were noted.

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ACKNOWLEDGMENTS

DECLARATION

The work contained in this thesis has not been previously submitted for any other qualification to any other higher education facility. To the best of my knowledge, this work contains no material previously published except where due reference has been made.

P. J. Lowe

Date   24/6/95

CHAPTER 1

INTRODUCTION

Annually, many people suffer unexplained fevers, headaches and fatigue, collectively known as pyrexia of unknown origin (PUO). In many cases, symptoms will resolve without medical intervention. Some cases, however, will require extensive periods of supportive therapy. The costs of medical intervention and the disruption of the patient's normal daily routine are shared by the community as a whole. In the spectrum of etiologic agents which may be associated with pyrexia, some cannot be directly treated (eg. Ross River virus) whilst with others (eg. Brucella), appropriate medical intervention can prevent long term morbidity and mortality; factors which are difficult to evaluate in monetary terms.

The initial step in the management of patients presenting with a PUO is to identify the etiologic agent causing the pyrexia. A detailed investigation of recent travel, occupation, recreational activities, animal contact, symptoms and duration of illness is an important aid in diagnosis and treatment. There is also a need to know the prevalence of the agents which may be associated with pyrexia in the community and in regions to which the patient has travelled. A presumptive diagnosis may then be made which will suggest appropriate laboratory testing and medical therapy.

Local studies of the prevalence of individual agents associated with pyrexia in the community are important in controlling health care costs and enhancing the clinical diagnosis of disease. This knowledge combined with the clinical presentation of the patient may reduce pathology costs by reducing the incidence of "blanket testing" which contribute to the burden on health care funding. Clinicians will be more adept in the diagnosis of disease if they are

aware of the clinical symptoms of organisms found in the community. Clinicians also need to be informed of new or previously unrecognised organisms causing infections in the community. Increasing travel and changing climatic conditions are allowing for the introduction of pathogenic organisms into new environments with previously unexposed hosts.

The Central Queensland Pathology Laboratory provides a service to a population of approximately 90 000 people. This includes regions south to the Sarina shire, west to the shires of Mirani and Nebo and north to the Whitsunday shire. The Mackay district has had few studies on the prevalence of pyrexial agents reported in the literature. The most recent study aware was published in 1953 by Derrick *et al* :- "Fevers of the Mackay District, Queensland" [1]. This study focused on the elucidation of the etiology of "Sarina Fever". Cases of scrub typhus, murine typhus, leptospirosis, Q fever and typhoid were recognised. There remained other fevers for which the diagnosis remained pyrexia of unknown origin. Retrospective analysis of laboratory reports to estimate prevalence data was considered inadequate because of the very low numbers of requests for serological testing of some of the agents of PUO to be included in this study. Some agents (eg. Barmah Forest virus and Lyme borrelosis) were unknown to many of the local doctors. This study was undertaken in an attempt to gain an insight into the prevalence of some of the agents which may be associated with PUO in this region in the 1990's. The list of agents associated with PUO selected for this study is by no means exhaustive. The organisms were chosen because of the availability of serological testing procedures, equipment and assistance from commercial suppliers of test kits and the State Health Laboratory in Brisbane, Queensland.

The Mackay district is well within the tropics being positioned at latitude 21° south. Average annual rainfall is 1636.9 mm, most of which falls in

the period January to April. The temperatures are quite moderate with the lowest recorded temperature in Mackay being 3.8°C on the 19th July 1965 and the maximum recorded temperature of 39.4°C on the 16th December 1964 (personal communication, Mackay Weather Bureau). The economy is largely based on the sugar industry but also has important cattle, mining and tourist industries. These tropical conditions and predominately rural lifestyle provide abundant opportunity for the existence of many zoonotic infectious agents, the diversity of which is only limited by the intensity of the search.

Accurate testing and notification of disease occurrence are important to establish the prevalence of agents of pyrexia. The predictive value of test results is directly related to the prevalence of the agent being assayed and therefore the results of inappropriate testing can be misleading if not considered in the correct context of each individual patient. Misdiagnosis of disease in its infancy can also be costly if the patient develops serious long term sequelae. The prevalence of pathogenic organisms will also influence preventative measures such as vector control, vaccination and community awareness. Control methods can be linked to seasonal variation and epidemics may be identified early and control measures implemented to minimise the numbers of people affected. The identification of patients with disease not previously recognised in the community has important consequences for future testing protocols.

Ross River Virus

RRV belongs to the family *Togaviridae*, genus *Alphavirus*. The virions are 60 to 70 nm in diameter and contain a single stranded RNA genome [2]. The Alphavirus group together with the Rubiviruses are responsible for most of the arthritic complications of viral infections [2].

The first descriptions consistent with the clinical manifestations of RRV disease were documented in 1928 [3]. Although the term "epidemic polyarthritis" was coined in 1946 [4] to describe the infection, but an the virus was not isolated until 1963 (Doherty *et al*,1963) from mosquitoes collected from Ross River near Townsville [5]. Serological evidence of RRV infection as well as viral isolates have been obtained throughout Australia and in Papua New Guinea, Indonesia, Solomon Islands, Fiji, New Caledonia, Samoa, Tonga and the Cook Islands.

The major vectors for RRV in coastal regions are the salt marsh mosquitoes *Aedes vigilax* and *Aedes camptorhynchus* [6]. These mosquitos breed in pools and saline marshes filled by tidal waters. *A. vigilax* activity peaks in the summer months but has intraseasonal cycles influenced by tidal variations. Rainfall appears to be of secondary importance in the breeding of *A. vigilax*. *A. camptorhynchus* has peak activity in winter and spring. *Culex annulirostris* is the principle inland vector, breeding in pools and swamps [6]. Seasonal cycles are regulated by temperature and intraseasonal cycles are influenced by rainfall. Epidemics of RRV infection are associated with human - mosquito - human cycles in times of increased vector activity. Maintenance of this cycle requires the presence of individuals with high viraemias titre.

Animal hosts for RRV are important as reservoirs for human infection. Antibodies against RRV have been detected in kangaroos, wallabies, bandicoots, horses, cows, feral pigs, foxes, hog deer and dogs [6]. In a serological survey conducted by Campbell *et al*, 50% of all horse sera and 74% of Grey kangaroo sera tested positive for antibody to RRV [7]. There is some suggestion that RRV infection in horses may be associated with muscle and joint stiffness and swelling [6]. The importance of animal hosts for maintaining a reservoir for RRV is illustrated by the fact that since the epidemic in Fiji in 1979, there has been a lack of reported cases indicating that a suitable animal reservoir does not

exist [8]. Following the epidemic, up to 90% of the population had antibody to RRV which precludes any further epidemics in the immediate future [8].

RRV infection is often subclinical as evidenced by the large number of seropositive patients in endemic regions [9]. The ages of affected people also varies widely. In the 1979 epidemic in Fiji, patients ranged from 4 to 80 years of age [8]. For reasons which are not clear, symptomatic RRV infection is uncommon in children [9,10]. Before the outbreak in Fiji, it was established that there was only a low level of alphavirus activity, but during the outbreak, children were significantly less affected than adults [8]. This would suggest that the presence of maternal antibody is not the sole reason for this fact. The disease was also more commonly reported in females than in males. In the study by Aaskov *et al* reported in 1981 it was noted that the female to male ratio in northern Queensland was approximately equal whilst in the shire of Fitzroy in central Queensland the female to male ratio was approximately two to one [9]. The differences reported may be due to strain variations of RRV. A viral isolate from the Fiji epidemic was thought to be different from the prototype T48 strain of RRV [8]. Isolates of RRV collected from north Queensland have been shown to be different from those collected from Nelson Bay in New South Wales [6].

Polyarthritis is the typical clinical presentation of RRV disease. Arthritis usually develops rapidly and most commonly occurs in the wrist, followed by the knees, ankles, fingers, elbows, toes, shoulders and hips [6, 8, 10, 11]. Clinical presentations may also include a rash which can appear 11 days prior to and 15 days after the onset of arthritis [12]. The rash occurs mainly on the limbs and trunk but can also occur on the extremities. It usually presents as erythematous macules and papules one to five mm in diameter and may persist for months although it usually resolves in one to ten days. In some patients, the rash may

only be evident as discrete macules on the webbing between fingers, contributing to the difficulty in diagnosing the disease based on the presence of a rash alone. Myalgia, fatigue, chills, fever, headache and lymphadenopathy also occur. Excessive fatigue after exertion often persists after other symptoms have subsided [10].

Antibodies to RRV may be detected after the viremic stage of infection. Antibodies of the IgM class may be detected by enzyme immunoassay (EIA) about one week after the onset of symptoms and usually decline to undetectable levels after two to three months [13]. Persistence of IgM does occur in some individuals leading to problems in diagnosis in non-epidemic situations [13]. Antibody levels in sera collected two weeks apart (acute and convalescent) should be examined for four-fold titre changes to indicate recent infection [14]. Results on single serum samples should be interpreted in the light of clinical presentation and onset date of symptoms. Clinical symptoms often persist long after the disappearance of IgM making the diagnosis of recent infection with RRV difficult [14]. IgG titres peak about four weeks after the onset of symptoms and then slowly decline, persisting for life [13]. Virus may be isolated from patients during the first week of illness before the production of specific antibodies.

The pathogenesis of RRV infection is poorly understood. Long term survival in synovial tissues in quantities undetectable by present techniques is a possible explanation for the persistence of symptoms [15]. This line of thought is supported by the predominately mononuclear cell infiltrate seen in joint fluid and in biopsies of rash. The absence of detectable circulating immune complexes [16], and the predominately mononuclear response to infection suggest an occult foci of infection with a predominantly cellular immune response [15].

Leptospirosis

Leptospirosis is a world-wide zoonosis with diverse clinical findings caused by infection with one of the 212 recognised serovars of *Leptospira interrogans* [17]. *L. interrogans* is a spirochaete 10 to 20 μm long and 0.2 μm in cross section. The most severe form of leptospiral infection, Weil's disease was described by Weil in 1886 [17]. The causative organism was discovered in 1915 and classified in 1918 as *Leptospira icterohaemorrhagiae* [18]. Swineherd's disease which is more common in Australia and Europe was described as a disease due to infection by *Leptospira pomona* in 1942 by Derrick [18].

Animal hosts of leptospira includes cattle, pigs, horses, dogs, rats, bandicoots and other small marsupials. Recognised associations between specific animals and specific serovars include serovars *hardjo* with cattle, *pomona* with pigs, cattle and rodents, and *icterohaemorrhagiae*, *copenhageni*, *bataviae*, *autumnalis*, *australis* and *javanica* with rats and rodents. These organisms may persist in the renal tubules of animals after clinical recovery providing an avenue for persistence of disease in the environment. The renal carrier state is central to the persistence of leptospirosis [19]. Leptospirosis occurs most commonly in abattoir and livestock workers. Occupational exposure to infected herds is a year round problem. Recreational activities which lead to direct contact with infected animals, contaminated soil or water is typically seasonal due to the inability of leptospiras to remain viable in dry, acidic environments (pH less than 7.0) [19]. Animals that produce an alkaline urine (herbivores etc.) are more important as shedders of leptospires than are those animals that produce an acid urine [19]. Leptospires enter through breaks in the skin. Prevention of infection relies on avoidance of contaminated materials, reduction of rodent numbers and vaccination of domestic animals and livestock [19].

The symptoms of leptospirosis range from being completely sub-clinical, to a flu-like illness, through to the severe icteric illness associated with Weil's disease. Infection during pregnancy carries the risk of intrauterine infection and foetal death [19]. In livestock, congenital infection may cause abortion, stillbirth, loss of milk production and failure to thrive [19]. The average incubation period is seven to ten days with the onset of symptoms being typically abrupt and including fever, myalgia, headache, chills, haemorrhage, transient rash and lethargy. Conjunctival suffusion (red eyes) and muscle pain are the most characteristic physical signs in this period of leptospirosis. In some patients, the muscular pain in the abdominal region has been so severe that inappropriate surgical intervention has occurred (eg. appendectomies) [17].

Antibodies begin to appear about two to three weeks after contact with leptospira. The antibodies form complexes with the leptospiras, are trapped in the kidney and are excreted in the urine for two to four weeks [19]. The anti-leptospiral antibody in urine does not kill the leptospiras, which may be cultured from urine at this stage if the sample is cultured immediately [19]. Immunity is humoral, and infection with a different serovar will result in an increase in antibody specific for the new infecting serovar [19].

The reference technique for leptospira antibody determination is the microscopic agglutination test (MAT) [19]. This test is based on the principle that live leptospiral antigens in the presence of antibodies in the serum agglutinate. The endpoint titre is determined using darkfield microscopy and is the highest dilution of patient serum showing a 50% agglutination. A fourfold rise in titre between acute and convalescent sera or a single high titre of 400 or greater is considered evidence of recent infection. Using a range of leptospira serovars in the MAT enables the laboratory to determine the identity of the infecting leptospira. Because this test utilises a battery of live leptospira serovars, the

MAT is usually only performed in reference laboratories. Other testing procedures include the complement fixation test (CF), EIA and the macroscopic slide agglutination test (MSAT). These testing procedures provide smaller laboratories with the opportunity to screen patient sera and refer any positive or doubtful results to laboratories that perform the MAT. This reduces the burden on reference laboratories by reducing the number of negative sera to be screened by the MAT as well as increasing the turn around time for the results of negative tests.

Laboratory findings in patients with leptospirosis include elevated skeletal muscle enzymes in about half the cases. Jaundiced patients have elevated serum bilirubin levels without markedly raised transaminases. Serum amylase may also be raised. There is generally a leucocytosis and the clinical impression of aseptic meningitis is presented in most patients.

The bacteremic stage of leptospirosis lasts about seven days. During this stage, leptospira organisms circulate through the blood stream until specific antibodies form complexes. Leptospiral endotoxin induces lesions in the endothelium of small blood vessels resulting in renal tubular necrosis, hepatocellular damage, meningitis and myositis [19]. Although the functioning of the liver and kidney may be severely impaired, histopathological lesions are not pronounced. Pulmonary manifestations and respiratory complications may also occur. All of these sequelae are reversible and are followed by complete repair upon resolution of the disease.

Antibiotic treatment is effective in the first seven to ten days of infection. Antibiotics shown to be effective in experimental infections include penicillin, ampicillin, tetracyclines, erythromycin, some third generation cephalosporins and some quinolones [19]. The regime of choice is five MU

benzylpenicillin injected daily for five days. Erythromycin may be given at dose of 250 mg 4 times daily for 5 days in patients allergic to penicillin. The Jarisch-Herxheimer reaction is well recognised in other spirochaetal infections but its occurrence in leptospirosis has been debated. After therapy, body temperature rises and falls and pre-existing lesions and clinical features are exacerbated. A Jarisch-Herxheimer reaction has been described for patients treated with penicillin [20]. Treatment is symptomatic but fear of a Jarisch-Herxheimer reaction should not prevent administration of appropriate antimicrobial therapy in suspected leptospirosis cases.

Flavivirus Infections

The flavivirus group are taxonomically group B arboviruses in the family *Flaviviridae*, genus *Flavivirus*. They are spherical single stranded RNA virions measuring 40 to 50 nm in diameter. More than 70 viruses have been assigned to this genus and classification schemes have been proposed according to antigenic relatedness. Generally, mosquito-borne flaviviruses are far more antigenically similar to one another than they are to the tick-borne flaviviruses or the flaviviruses with unknown vectors. It has long been recognised that sera from patients with flavivirus infections may react with several distinct antigen sources. This means that laboratories using serological assays must recognise the limitations of the results. For instance, positive antibody results do not exclude the possibility that the infecting agent is a virus closely related to the one presumptively identified in the laboratory assay. In these situations, an accurate clinical history may help to determine the etiology of the infecting agent. There is also evidence of cross protection in infections with closely related flaviviruses. Allan *et al* have shown that T cells primed with West Nile virus responded to challenge form Kunjin virus and to a lesser extent Murray Valley encephalitis but had no response to challenge with dengue [21].

The flavivirus group of arboviruses have the potential for causing significant epidemics in susceptible populations in terms of mortality, morbidity and economic significance. The clinically most important flaviviruses in Australia are dengue, Murray Valley encephalitis, Kunjin, Kokobera, Edge Hill, Stratford and Alfuy. Cases of dengue fever have been recorded in 52 countries and the spread of the disease can be linked to the spread of *Aedes aegypti* [2]. Approximately 40 million cases of dengue fever occur world-wide annually. Dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS) is one of the most serious diseases known to man.

The clinically important flaviviruses in Australia have mosquito vectors. The principle vector for dengue is *A. aegypti*, and for Murray Valley encephalitis (MVE) and Kunjin, *C. annulirostris* is the principle vector. For Kokobera and Edge Hill, the vectors include *C. annulirostris* and *A. vigilax*, and for Stratford, *A. vigilax* is a known vector [22]. The major vertebrate hosts include waterbirds, cattle, some marsupial species and man [22].

Previous epidemics of flavivirus infection have been linked to increased vector activity in the warmer summer months associated with increased rainfall. Another factor important in the transmission of disease by mosquito vectors is the ability of the vector to produce high titres of virus. It has been shown that for *C. annulirostris* infected with MVE, reduction of the external temperature had the effect of reducing viral titres in mosquitoes. When mosquitoes were once again held at a higher temperature, viral titres were seen to be higher [23].

The clinical presentation of a patient with dengue fever following infection with either of the four serotypes is fever with or without a rash, myalgia and arthralgia [2]. Gastrointestinal bleeding may also occur in some patients. There is a window period of four to five days after initial infection

before development of symptoms. The development of a rash often signals the end of the disease in dengue fever. DHF/DSS occurs most often when patients with pre-existing antibody to dengue are infected with dengue virus of a different serotype [24]. After a similar incubation period, there is a sharp rise in body temperature which persists for two to seven days. Haemorrhagic manifestations include haemorrhages in the skin, subcutaneous tissues, gastrointestinal tract and heart. There is a leakage of plasma and plasma proteins into the extravascular space through vessel walls with no evidence of inflammation or necrosis. This is due to changes in capillary permeability, the severity of which may be related to patient age. From studies on outbreaks of DHF/DSS in Cuba, it appears that there is a correlation between the severity of the capillary permeability syndrome and age, with children under the age of 16 being most often affected [24]. In DHF/DSS, the onset of capillary permeability heralds the onset of the short period of acute physiological stress. Patients who survive do not have any long term sequelae whilst in fatal cases, death usually occurs in 24 to 48 hours. Thrombocytopenia is a major clinical finding in DHF/DSS. The dengue virus appears to exert a depressive effect on bone marrow elements, inhibiting marrow proliferation early in the course of the disease but not in the later stages [25]. Coagulation abnormalities also occur in DHF/DSS with prolongation of activated partial thromboplastin and prothrombin times and decreases in fibrinogen levels. The coagulation picture resembles a mild disseminated intravascular coagulation [24].

Murray Valley encephalitis is the etiologic agent of Australian encephalitis although some cases can be attributed to Kunjin virus infections [26]. Fever, headaches and encephalitis are the dominant clinical presentations and outbreaks of MVE have shown high case fatality rates. In 1974, 12 of 58 cases died whilst in 1951, 17 of 40 cases died [26]. Patients surviving MVE infections may be left with severe neurological sequelae. Kokobera virus has

been implicated in acute polyarticular disease of man [27].

Detection of antibodies to flaviviruses can be made with several techniques including haemagglutination inhibition (HI), complement fixation (CF), neutralisation (NEUT), immuno-fluorescent assay (IFA) and enzyme immuno-assay (EIA). The standard criterion for serological diagnosis is a fourfold rise or fall in antibody titre between appropriately collected paired sera [2]. Serum samples should be collected in the acute stage of illness and then after a period of two weeks. If an inconclusive rise in titre is determined, a third sample should be taken after a further two weeks. Antibody titres in samples collected late in the course of illness present more of a diagnostic dilemma as the levels may have already peaked and these levels tend to decline at a slower rate than that at which they rise. A confounding problem is the fact that broad cross-reactions may occur in HI, CF,IgM EIA and NEUT tests which may prevent the identification of the infecting agent [2]. Antibody already present due to a previous infection with another flavivirus may also mask antibody changes [2]. Infection with one flavivirus may result in antibody titre changes occurring to one or more related viruses to which the individual may or may not have been exposed [2]. Antibody cross-reactivity may broaden with time after infection [2]. EIA detection of antibody of the IgM class is possible soon after infection and unlike IgG antibody, does not usually persist for years. The detection of IgM antibody in a single serum sample has provided a rapid diagnostic tool for arboviral infections in epidemic situations. However, persistence of IgM has been found to occur in some individuals [2]. Results from patients in non-epidemic situations should be interpreted in light of the clinical presentations [14].

Lyme Borrelosis

B. burgdorferi is a typical spirochaete, loosely coiled with a left handed helix 18 to 30 μm in length and 0.2 to 0.3 μm in diameter. The organism is enclosed by an outer envelope of glycolipids and proteins. The flagella may vary in number and are internal, running parallel to the long axis of the organism [28]. The 41 kilodalton (kDa) flagellin subunit has been shown to have an amino acid sequence very similar to that of many other spirochaetes. There is heterogeneity in the major surface proteins between isolates of *B. burgdorferi* from around the world [28]. In general, North American isolates are much more homogeneous than isolates from Europe [28].

Lyme disease was first recognised in 1975 in Lyme, Connecticut during an outbreak of "juvenile rheumatoid arthritis" [29]. A total of 51 cases in this first outbreak included 12 adults and 39 children, all who lived in forested parts of the communities of Lyme, Old Lyme and East Haddam. Thirteen of the cases reported an erythematous papule that expanded into a red annular lesion with a partial central clearing. This description was consistent with cases of erythema chronicum migrans which had been associated with tick bites in Europe. This case description matched cases which had been noted as early as 1909 [30]. Lyme disease has been diagnosed in Australia in patients with a history of overseas travel [31].

In 1981, Burgdorfer noted spirochaetes in the digestive tract of adult *Ixodes dammini* ticks [29]. The spirochaetes were cultured and were found to react with sera from patients with Lyme disease. By the summer of 1982, spirochaetes had been isolated from Lyme disease patients and in 1984, the organism was named *Borrelia burgdorferi*. Today in the United States, Lyme disease is the most significant one tick-borne disease of humans [32].

B. burgdorferi was first isolated from the eastern deer tick, *I. dammini* collected from Shelter Island off the coast of eastern Long Island, USA [29]. On the west coast of the United States, the vector is the western blacklegged tick, *Ixodes pacificus*; in southern United States, *Ixodes scapularis* is the vector and in Japan, *Ixodes persulcatus* has been postulated as the vector [29]. In Europe, the principle vector is the European sheep tick, *Ixodes ricinus* [29]. All of these ticks have many animal hosts. Animal hosts of *B. burgdorferi* include mice, deer, raccoons, dogs, horses, cows, rabbits, birds and humans. Birds are important reservoirs because they are a natural means of distributing spirochaetes over long distances. In Australia, the logical vector is the paralysis tick *Ixodes holocyclus* [33]. The tick feeds predominantly on bandicoots but also attacks domestic animals and humans. The paralysis tick has a distribution right along the east coast of Australia. Recent studies on vector competence with American isolates of *B. burgdorferi* indicated that *I. holocyclus* was not a suitable vector but these studies should be repeated with an Australian isolate [33].

B. burgdorferi remains abundant in the midgut of Ixoide ticks and may cause a systemic infection with penetration of the tissues of the central ganglion, salivary glands, ovary and malpighian tubules [29]. Massive infections of oocysts have been found in these ticks and the destruction of the egg-forming process has prevented the female from ovipositing. Evidence for transovarial transmission comes from the discovery of infected unfed larval *I. dammini* suggesting that mild infections of oocysts still permit full development of eggs [29]. An occasionally observed phenomenon is the reduced staining intensity with the monoclonal antibody H5332 of *B. burgdorferi* from *Ixode* ticks with systemic infections in comparison to *B. burgdorferi* from midgut infections. This may represent an antigenic variation of the spirochaete in response to different or unfavourable growth conditions [29]. Reduced or complete lack of staining

intensity with H5332 has also been seen for some European isolates of *B. burgdorferi* [29].

Transmission of spirochaetes from ticks may occur by inoculation of spirochaete infected saliva [29]. One line of thought is that the spirochaetes pass through the gut wall, enter the salivary gland ducts and are flushed into the bite wound [29]. Periodic regurgitation of gut contents whilst feeding would also provide an avenue for the presentation of spirochaetes to open bite wounds [29]. This second method of transmission also gains support from the fact that less than five percent of adult *I. dammini* are systemically infected [29].

The classical clinical presentation of Lyme disease is erythema migrans (EM). EM is defined as a discernible, centrifugally expanding, usually erythematous annular patch. EM is observed in about 70% of patients and usually develops between 1 and 36 days after a tick bite [34]. The EM lesions are usually painless and nonpruritic. Histologic examination of skin lesions suspected to be EM is useful because the presence of spirochaetes can often be detected with Warthin-Starry staining [34]. In the early stages of Lyme disease, non-specific symptoms also occur and may include general malaise, myalgia, arthralgia, fatigue, headaches, fever and chills. This first stage of infection is often mild with many patients not presenting until months or years after initial infection.

Early in the course of infection, haematogenous dissemination occurs which may lead to multiple EM lesions and multi-organ tissue damage [35]. This second stage of infection may result in myocarditis and meningoencephalitis and may occur one to two months after initial infection. *B. burgdorferi* meningitis presents as an aseptic meningitis with pleocytosis and is often involved with facial palsies and peripheral nerve involvement [35].

The third stage of infection includes neurologic abnormalities, chronic encephalitis and development of arthritis [35]. The arthritis is characteristically episodic and primarily affects the larger joints especially the knee. There is usually a swelling of the joint caused by proliferation of synovial soft tissues with or without an increase in volume of synovial fluid. Spirochaetes have only rarely been seen in synovial fluid. A recurrence of EM has occurred in some patients with chronic Lyme disease [35].

Persistence of active infection may occur despite a measurable immune response [36]. Conversely, anti - *B. burgdorferi* antibodies do not necessarily reflect active infection. Individuals who are seropositive but asymptomatic may indicate a successful immune response to infection, or may indicate a false positive serological reaction [36]. Persistence of disease is not well understood. It may reflect successful evasion of the body's immune response, or may be due to some auto-immune response which has not yet been elucidated [36].

Diagnosis of Lyme disease in non-epidemic regions may rely solely on a clinical presentation of EM with an appropriate history of travel to an endemic area and/or a tick bite [37]. Following initial infection, there is an early T lymphocyte response followed by a slower B lymphocyte response. IgM class antibodies appear first followed by IgG antibodies [37]. The primary antibody response is to a 41 kDa flagellum antigen. IgM antibody can usually be detected by IFA and EIA three to four weeks after initial infection. IgG antibodies may be detected 8 to 12 weeks after initial infection. IgM levels peak at about eight weeks and usually decline although some persistence has been shown to occur.

Serological assays for Lyme disease suffer from problems with false negatives due to the fact that not all patients with clinical disease develop antibodies at high enough titres to be detected in the current IFA and EIA test

systems [37]. The current serological assays use whole spirochaete preparations which do not detect the early rise in antibody titre above the background of cross-reacting antibodies. False positive results may occur because the 41 kDa flagellum associated antigen is in part homologous to antigens found in *Borrelia recurrentis*, *Borrelia hermsii* and *Treponema pallidum*, the causative agents of relapsing fever, louse-borne relapsing fever and syphilis respectively [38]. Some cross-reactivity has also been seen for patients with Rocky Mountain Spotted Fever [38]. Serological assays have increased positive predictive values when detecting antibodies in the latter stages of infection due to the higher antibody titres [37]. In chronic infections, IgM antibody may be detected which limits the usefulness of this antibody class as a marker for acute infection [37].

The optimal treatment regimen for *B. burgdorferi* infections has not been established. Studies in clinical disease, animal models and *in vitro* have shown *B. burgdorferi* to be susceptible to penicillin G, ampicillin, amoxicillin, tetracycline, erythromycin, ceftriaxone and cefotaxime. When considering chemotherapy and its duration, the rapid haematogenous dissemination of spirochaetes which may include CNS invasion should be bourne in mind. Therapy must therefore include coverage for possible early neurological involvement. Amoxicillin and doxycycline are suitable forms of therapy in the early stages of disease without evidence of CNS involvement. For the more serious complications of arthritis and CNS disease, third generation cephalosporins such as ceftriaxone and cefotaxime should be used.

Barmah Forest Virus

BFV is an alphavirus. The virion is enveloped and has a diameter of approximately 50 nm containing a single stranded RNA genome. Antibodies to BFV weakly cross-react in HI tests with other alphaviruses such as Sindbis and

RRV but not in neutralisation or complement fixation tests [39]. Unlike a number of other alphaviruses such as RRV, Getah, Semliki Forest and Western Equine encephalomyelitis, BFV was found not to cross the placenta in laboratory infected mice [40]. The authors however noted some limitations in the experimental protocol which will need further evaluation to exclude the possibility of transplacental transmission.

BFV is named after the forest bordering the banks of the Murray River in northern Victoria. The virus was first isolated from *C. annulirostris* mosquitoes collected during the summer of 1974 [41] in Barmah Forest and at about the same time from mosquitoes collected in Queensland [42]. Antibodies against the virus in human sera were first detected in 1986 [43] and since that time, serological surveys have suggested many sub-clinical infections at the rate of 0.23% of the population per annum in Queensland [44]. BFV was first isolated from a patient, in Queensland, in 1989 [44].

BFV has been isolated from *C. annulirostris*, *A. vigilax*, *Aedes normanensis*, *Aedes bancroftianus*, *Coquillettidia linealis* mosquitoes and the midge *Culicoides marksi* [45]. Vertebrate hosts other than humans are unknown at this stage as is the life cycle and possible seasonal variations of viral infections.

The clinical presentations of infection are similar to those seen in RRV disease with the main symptoms being arthritis/arthralgia, myalgia, fever and rash. The joints most commonly affected are feet, knees, wrists and elbows [44]. The duration of symptoms is unknown but in the 1988/1989 study, 8 of 19 patients had recovered within 8 weeks of onset of symptoms [44].

Antibodies to BFV may be detected by HI and EIA. The onset and duration of IgM are not known but in the Queensland study, IgM was detected at

the onset of symptoms in one patient and within three days of onset of symptoms in a further nine patients [44]. Cross-reactions in HI may occur with other alphaviruses but generally do not occur at equivalent titres. IgM antibody to alphaviruses is thought not to cross-react [2]. Data regarding immunity to reinfection and protection by maternal antibody is lacking.

Brucellosis

Brucella are short gram negative bacilli measuring 0.5 to 0.7 μm by 0.6 to 1.5 μm. Growth is optimal at 37°C in an aerobic atmosphere with some strains requiring an increased carbon dioxide level for primary isolation [46]. Currently listed are six species of brucella: *Brucella melitensis*, *Brucella abortus*, *Brucella suis*, *Brucella canis*, *Brucella ovis* and *Brucella neotomae*. *B. ovis* and *B. neotomae* do not normally cause infections in man [46].

Brucellosis was first recognised as "Malta fever" which affected British troops based in Malta at the beginning of the 18th century [47]. Soldiers were affected with persistent high fever and pain in the arms and legs. The first isolation of the causative organism was made by David Bruce in 1883 from the spleen of a soldier who had died of Malta fever [47]. Epidemiological studies linked Malta fever with consumption of sheep and goat milk in which *Brucella spp.* were isolated.

Brucellosis is a zoonotic disease usually associated with contact with domestic animals or their food products. *B. melitensis*, *B. canis*, *B. abortus*, *B. suis*, *B. ovis* and *B. neotomae* infect sheep and goats, dogs, cattle, pigs, sheep and desert rats, respectively [46]. Infections occur globally and are usually associated with occupational exposure (livestock handlers, abattoir and dairy workers etc.) or ingestion of contaminated food products (milk and cheese) [46]. Infections arise

from direct contact with viable organisms with the mucosa of the gastrointestinal tract or conjunctiva or through abrasions of the skin [46]. The organisms enter the lymphatic system and multiply in lymph nodes unless destroyed by the cellular immune response. A bacteraemia then develops which may lead to the establishment of many foci of infection in tissues and organs including the liver, spleen, bone marrow, heart and other lymph nodes [46].

The incubation period is typically 10 to 14 days. In acute cases, fever often presents in late afternoon or evening with temperatures ranging from 38°C to 40°C. Brucellae multiply in the phagosomes of polymorphonuclear leucocytes which protects them from serum antibody and some antibiotics. Endotoxin is released gradually into the blood which produces the characteristic relapsing fever. Other symptoms include myalgia, headache, sweating, weakness, malaise, weight loss, hepatomegaly, splenomegaly, coughing, vomiting and arthralgia [48]. In chronic brucellosis, abscesses may develop in subcutaneous tissue, testis, epididymis, ovary, gall bladder, kidney and brain [46].

Definitive diagnosis is made by isolation of the infecting organism. Blood cultures should be incubated with increased carbon dioxide for up to 30 days. Isolation from exudate and tissue material is made by plating onto blood agar, chocolate agar, Brucella agar or serum dextrose agar and incubating for ten days at 37°C in an atmosphere of 5 to 10% CO_2. Identification of isolates is made by agglutination in monospecific sera, urease production, requirement for increased CO_2, production of H_2S, growth in the presence of basic fuchsin and thionin in solid media and an oxidase positive reaction [46].

Serological diagnosis of infection is complicated by numerous cross-reactions occurring in patients affected by *Francisella tularensis*, *Vibrio cholerae*, *Yersinia enterocolitica* serogroups O:9 and O:16, *Escherichia coli* serogroup O:157

and *Salmonella* serogroup O:30 [46]. IgM antibody titres may persist for months or years without ongoing infection. Following treatment, IgG levels fall more quickly than IgM and may be a useful indicator of successful therapy [49]. Serological tests include the serum agglutination test (SAT), CF, indirect coombs test, radioimmuno-assay and EIA. The SAT is considered the standard for serological diagnosis of brucella infection [49]. In populations not commonly exposed to brucella, a titre of 1:160 or greater in a single serum sample or a fourfold rise in titre is considered significant [49]. Abattoir workers, veterinarians or other animal handlers may have higher background levels of antibodies and so titres in the range of 1:320 to 1:640 are considered significant [49]. Treatment of sera with 2-mercaptoethanol (2ME) causes the IgM molecule to lose agglutinating activity and thus provides a means for measuring agglutinating IgG [49]. The 2ME SAT results may help to confirm chronic infections and also provide an indication of successful treatment [49].

The World Health Organisation recommends oral tetracycline or doxycycline plus intramuscular inoculation of streptomycin or gentamicin for treatment of acute brucellosis. In adults, chemotherapy with doxycline plus rifampin or cotrimoxazole given orally has proved successful [50]. Surgical drainage of abscesses is indicated as is valve replacement in endocarditis cases.

Rickettsial Infections

All rickettsia are included in the family *Rickettsiaceae* order *Rickettsiales*. They are gram negative, obligate intracellular bacteria measuring 0.8 to 2.0 μm long by 0.3 to 0.5 μm wide [51]. Rickettsia with the exception of *Coxiella burnetii*, are unstable outside the host cell and are inactivated by temperatures above 56°C and by disinfectants [51].

Rickettsial infections take many forms with the oldest being epidemic typhus. Typhus has spread worldwide largely due to war and the mass movements of infected persons. The significant effects of typhus are borne out by the historical reviews of war. In 1489, Ferdinand the Catholic lost 20 000 soldiers of which 17 000 died from typhus [47]. The battle of Nuremberg, in the Thirty Year's War, could not take place because of an outbreak of typhus on both sides [47]. More recently, German refugees expelled from the East in the winter of 1944/45 brought typhus to the West which claimed many lives [51].

Charles Nicolle was the first person to incriminate the louse as the vector of typhus with transmission experiments from sick to healthy monkeys in 1909 [47]. Howard Taylor Ricketts and Stanislaus von Prowazek contributed much towards an understanding of the epidemiology of typhus, a disease from which they themselves died [47]. The causative organism *Rickettsia prowazekii* was named in honour of these two pioneers [47].

Today there are five recognised groups of rickettsia spread by ticks, mites, lice and fleas. These are the Spotted fever group, Typhus group, Scrub typhus, Q-fever and Trench fever. The causative agent of Queensland Tick Typhus (QTT), is a member of the spotted fever group and was first described in 1946 during an outbreak amongst soldiers training in north Queensland [52]. The name *Rickettsia australis* was first used in 1950 as the etiologic agent for QTT. The name QTT has persisted despite the fact that many cases occur not only in the coastal regions of Queensland, but also along the coast of New South Wales and Victoria. An outbreak of a spotted fever-like illness on Flinders Island, Tasmania was reported in 1990 and had been presumed to be due to *R. australis* infections [53]. However recent DNA nucleotide sequence analysis has shown that an isolate from Flinders Island was quite distinct from *R. australis* [54].

The spotted fever group includes *Rickettsia rickettsii*, *Rickettsia sibirica*, *Rickettsia conorii*, *R. australis*, *Rickettsia akari* and *Rickettsia japonica*. *R. prowazekii* and *Rickettsia typhi* comprise the typhus group, while *Rickettsia tsutsugamushi* ,*C. burnetii* and *Rochalimaea quintana* are the causative agents of scrub typhus, Q fever and trench fever respectively [51]. *R. australis* is transmitted to humans by the paralysis tick *I. holocyclus* [53]. This tick is distributed along the east coast of Australia and is an indiscriminate feeder on a variety of animals including man, dogs, bandicoots, kangaroos and birds. Adult ticks are most numerous in the months August to December which causes some seasonal variations in disease occurrence. *R. australis* has also been isolated from *Ixodes tasmani* but its role as a vector is not understood as this tick rarely bites humans [53]. *I. tasmani* is more widely distributed, being found away from eastern coastal regions and in Western Australia. Tick bites on Flinders Island have been attributed to *I. tasmani* and thus may prove to be the vector of spotted fever group infections in this location [53].

Rickettsia enter host cells by induced phagocytosis, escape the phagosome and multiply within the cytoplasm of the cell [51]. The host cell eventually ruptures and releases rickettsia to invade surrounding cells. An invasion of the capillary vascular epithelium occurs which may cause swelling and inflammation at the inoculation site. An eschar may form at the site of the tick bite. After proliferation at the site of infection, rickettsia disseminate into the vasculature of many organs causing lesions by destruction of intracellular membranes [51]. Damage to the endothelial cells of the vascular systems causes petechial lesions and may cause lesions in the brain, kidneys, lungs and heart [51].

The clinical manifestations of QTT are typically abrupt in onset and include fever, headache, myalgia, arthralgia and some nausea or vomiting. In the

series of patients studied by Sexton *et al* [53], a skin rash occurred as early as day 1 and as late as day 12 after onset of symptoms. Of the patients in their study, 2 of 62 failed to develop a rash. Descriptions of the rash varied from chicken pox - like to blotchy and sparse or maculopapular. The palms and soles were frequently affected. The severity of illness varied from mild to fatal.

Definitive diagnosis of infection can be made by isolation of the infecting organism. Rickettsia are extremely hazardous and so isolation or cultivation of organisms should only be attempted in laboratories with biosafety level three containment facilities [51]. There had only been six isolates of *R. australis* from humans and ticks until December 1990 [53]. Rickettsial organisms may be visualised in direct biopsy specimens with the use of direct fluorescent antibody tests but this procedure generally lacks sensitivity [51].

Historically, detection of antibody following rickettsial infections was made using Weil - Felix tests [51]. In the 1920's, it was observed that certain strains of the bacterium, *Proteus* would agglutinate early convalescent sera from patients with rickettsial infection [51]. QTT infected patients may show rises in antibody titres against *Proteus* OX2 or OX19. The Weil - Felix test is considered to be insensitive and not specific for rickettsial infections and should not be used as a stand alone test for diagnosis. CF tests are also considered to be insensitive [51].

Newer test procedures include IFA, EIA, RIA and latex agglutination. Apart from the IFA test, reagents are usually only available in reference laboratories. In diagnosing rickettsial infections, use can be made of the fact that sera from patients infected with any species of rickettsia will react with rickettsial antigens of any other species in that serogroup [51]. In the IFA test, IgM is usually detectable in six to ten days after onset of symptoms and usually

declines after ten weeks whilst IgG can be detected two to three weeks after onset of symptoms and usually declines after one year [51]. The Centres for Disease Control in the United States of America have established criteria for serological diagnosis in IFA tests [51]. Single titres ≥64 are considered diagnostic for spotted fever group infections [51]. A fourfold rise in titre is considered evidence of rickettsial infection [51].

Therapy is usually based on one of the tetracyclines or chloramphenicol. Prompt therapy has been shown to reduce morbidity and mortality even though these agents are rickettsiostatic and not rickettsiacidal. In the series studied by Sexton *et al* [53], 43 patients recovered without antimicrobial treatment whilst 31 patients required hospitalisation.

Streptococcal Infections

Streptococci are gram positive cocci, which on the basis of specific cell wall associated carbohydrates, have been divided into 13 serogroups designated A to O. Streptococci cultured on sheep blood agar can be classified on the basis of the pattern of haemolysis they produce. α-haemolytic streptococci produce a zone of partial haemolysis of the red blood cells in the agar to produce a greenish tinge in the agar. ß-haemolytic streptococci lyse the cells due to the action of haemolysins and produce clear zones around individual colonies. Non-haemolytic streptococci are termed δ-haemolytic.

Streptococci may well be the most important bacterial pathogens of humans as they have the ability to invade any tissue and produce many different clinical syndromes [55]. These include throat infections, lymphangitis, puerperal fever, rheumatic fever, erysipelas, acute glomerulonephritis and polyarthritis. Descriptions consistent with scarlet fever were first noted in the

fifth century BC by Hippocrates [47].

Streptococci produce several extracellular products to which the body in turn produces antibodies. These antibodies do not protect against streptococcal re-infection. Streptolysin O, a haemolysin, is produced by group A streptococci and also by some group C and G streptococci [55]. Raised antistreptolysin O (ASO) titres can be detected in approximately 85% of group A respiratory infections, in approximately 90% of patients with acute glomerular nephritis following streptococcal pharyngitis and in only about 25% of patients with glomerular nephritis following streptococcal pyoderma [55]. Titres greater than 200 may be considered significant in patients over 5 years old [55]. In children less than 5 years of age, titres of 50 or more may be considered significant [55]. Group A streptococci can produce four distinct deoxyribonucleases; A, B, C and D. DNase-B is the form found most consistently. Antibodies to DNase-B (ADB) have a normal upper limit titre of about 170 depending on the population being screened. Significant ADB titres are found in approximately 80% of the cases of rheumatic fever, in approximately 60% of cases of streptococcal pyoderma and in approximately 75% of cases of streptococcal pharyngitis [55].

Other extracellular products include the erythrogenic toxin responsible for the rash in scarlet fever, streptokinase which catalyses the conversion of plasminogen to plasmin and hyaluronidase which may be responsible for connective tissue damage in serious streptococcal infections. Titres are usually determined for at least two of these products to increase the chances of detecting streptococcal infections of group A etiology. Diagnosis should be made utilising appropriately collected acute and convalescent sera and demonstration of a four-fold rise in titre [55]. Unfortunately, most patients do not present in the acute phase of infection and thus titres may have already peaked. High ASO and ADB results should be interpreted in light of the clinical presentations as

these levels may remain elevated for considerable periods. In a study of rheumatic fever in children in Japan, the ASO titre was found to be elevated for an average 4 months after infection whilst the ADB titres did not return to normal for an average of 35 months [56].

The treatment of choice for group A streptococcal infections is penicillin. The site and seriousness of infection dictates the length of treatment. Erythromycin may be used in patients allergic to penicillin.

Rheumatoid Factor

Rheumatoid arthritis (RA) is a chronic inflammatory disease of unknown etiology. Usually, peripheral joints are affected in a symmetrical distribution. Diagnosis of RA is made on a combination of clinical and radiographic findings with or without the presence of detectable rheumatoid factor (RF) [57]. RF can be found in healthy individuals and in individuals afflicted with a variety of diseases including mononucleosis, hepatitis, influenza, tuberculosis, syphilis, brucellosis and subacute bacterial endocarditis.

RF, first discovered in the 1940's, is an antibody specific for IgG and may be either an IgA, IgM or IgG isotype [57]. IgM-RF is the main isotype measured in diagnostic assays [58]. Synthesis of RF appears to be related to chronic antigenic challenge [59]. It appears that RF may have a role in the capture, processing and presentation of antigens trapped in immune complexes to T cells [59]. Studies have shown that patients with subacute bacterial endocarditis develop RF which disappears upon successful treatment of infection [57].

Aims and Objectives

This study was undertaken in an effort to determine, by serological methods, the prevalence of a number of infectious organisms with similar clinical presentations. The impetus for this study was the large number of patients being serologically tested for evidence of RRV infection for whom the results suggested no evidence of exposure to the virus despite the presence of acute symptoms often associated with RRV infection. The question that was often posed by the doctor was "what else could be responsible for these symptoms in the patient?" The possibility existed that other infectious organisms were being overlooked as a diagnosis in favour of a more "popular" disease such as RRV infection.

A study such as this also allows the prevalence of newly recognised infectious organisms such as BFV and lyme borrelia to be determined from this subset of the central Queensland population. The prevalence of "older" organisms such as leptospira, brucella and rickettsia could also be determined for the Mackay district. It is hoped that the results from this study will enable improved disease diagnosis and patient management. This should also aid in the reduction of health care costs.

It should be noted that the prevalence data obtained from a study such as this utilising "sick" patients as a sample population may not accurately reflect the population as a whole. The available resources however, did not allow random population sampling to be undertaken. The results obtained from this study may indicate the need for further studies to be undertaken utilising a broader population base.

CHAPTER 2

METHODS AND MATERIALS

In this study, the prevalence of pyrexial agents in the Mackay district was determined by serological methods utilising patient sera submitted to the Central Queensland Pathology Laboratory for Ross River virus (RRV) serology testing. Included in the study were tests for the detection of antibodies to RRV, Barmah Forest virus (BFV), flavivirus, leptospira, spotted fever group rickettsia, brucella and lyme borrelia. Titres of rheumatoid factor and streptococcal antibodies were also determined. Of the infections and agents listed, only the prevalence of antibodies to RRV could be determined with any accuracy before the study as the request numbers for serology for any of the other agents were too low to be statistically significant. Lyme borreliosis and BFV infections were (and still may be) new clinical entities for some local clinicians and so the study provided a good opportunity for education in two respects. Clinicians were made aware of the clinical presentations of these diseases and laboratory staff gained knowledge of the prevalence of these organisms in the community.

This project was carried out over the period January 1991 to December 1992. Patient sera were systematically sampled by utilising samples submitted for RRV serology during the first week of each month. Serum samples were stored at minus 20°C if not processed within 24 hours. Additional testing requests and any clinical history was noted from the patient's pathology request forms. All patient results were treated with due confidentiality and any significant results from testing not specifically requested were related to the referring doctor by phone.

RRV IgG and IgM antibody determinations were made using the PanBio ELISA test kits (PanBio, Brisbane, Queensland, Australia). The kits were used

according to the manufacturer's instructions. These test kits are an indirect enzyme immunosorbent assay using a sheep anti-human IgG precipitating solution in the IgM assay to avoid problems with competing RF and IgG. Patient sera were assayed at a 1:100 dilution and were incubated at room temperature in wells of a microtitre plate coated with RRV antigen for 20 minutes. Following washing, anti-human conjugate was added to each well. The microtitre plate was incubated at room temperature for 20 minutes. After washing, a tetramethybenzidine/hydrogen peroxide substrate was added. The reaction was stopped with the addition of acid. Absorbances were measured photometrically. The results were determined by calculating the average absorbance of a positive calibrator and multiplying the result by a conversion factor supplied with each kit. The absorbance value of the patient's serum was divided by this figure. Values less than 0.9 were considered negative. Results in the range 0.9 to 1.1 were considered equivocal and were repeated. Values greater than 1.1 were considered positive.

Rickettsial serology was performed using IFA. Advantage was taken of the fact that members of the spotted fever group of rickettsia cross-react in immunoassays [51]. Commercial slides coated with *R. conorii* antigen were purchased from Sanofi Diagnostics Pasteur (North Sydney, Australia). Positive control sera were kindly provided by Brian Dwyer (Fairfield Infectious Diseases Hospital, Melbourne, Australia). The assay was performed as per the manufacturer's directions with the following exception. All sera for IgM testing were treated with GullSORB (Gull Laboratories, USA) as per the manufacturer's instructions to remove competing IgG. Diluted patient sera was incubated on separate spots on the IFA slide for 30 minutes at 37°C. After washing with phosphate buffered saline, 30 uL conjugate (IgG or IgM) was added to each spot and incubated for a further 30 minutes at 37°C. After washing, the slide was coverslipped and read with a fluorescent microscope. A test was considered

positive on the basis of very bright, apple green dots when viewed with the fluorescent microscope. Negative sera showed no fluorescence.

Leptospira serology was performed using the macroscopic slide agglutination test kit from Sanofi Diagnostics Pasteur. This test kit utilises a heat stable leptospira antigen which is claimed to agglutinate with antibodies to any of the serovars of *Leptospira interrogans*. Patient sera were diluted one in two with physiological saline, mixed with antigen and rotated for four minutes on a microplate mixer. The results were determined using low magnification darkfield microscopy to observe agglutination. Positive control sera were provided with each kit. Any results considered to be doubtful or positive were referred to the Queensland State Health Laboratory which is the World Health Organisation Australian Leptospirosis Reference Laboratory. In this laboratory, the specimens were assayed using the standard MAT and CF tests.

Brucella serology was performed using Stained Brucella Suspensions from Murex (formerly Wellcome Diagnostics, Sydney, Australia). Patient sera were screened using the protocol for the rapid screening test as per the package insert. Briefly, 20 uL and 10 uL of undiluted serum was added to circles on a white tile. One drop of appropriate suspension was added to each circle. Stained suspensions of *B. abortus* and *B. melitensis* were used in the assay. Any agglutinations were confirmed using the tube agglutination test procedure to determine the antibody titre. In this procedure, a row of serial dilutions, starting from 1:20 for each antigen to be tested was made to a volume of one mL in clean test tubes. A drop of the appropriate suspension was added to each tube. The suspensions were mixed and incubated for 48 hours at 37°C. Positive tubes have obvious granular agglutination. The titre is recorded as the tube with the highest dilution showing agglutination.

Streptococcal serology was performed utilising two assays. The ASO titre was determined using reagents from Murex. A 1:10 and 1:50 dilution of patient serum was made in buffer. In a standard microtitre plate, 50 uL of buffer was added to wells B to H in columns 1 and 2. A 50 uL aliquot of the 1:10 dilution was added to wells A and B in column 1 and 50 uL of the 1:50 dilution was added to wells A and B in column 2. Using a multichannel pipette, 50 uL was serially diluted in columns 1 and 2 from B to H and then discarded. To all wells, 25 uL of reduced streptolysin O was added. The plate was covered and incubated for 15 minutes at 37°C. Following this, 25 uL of 3 to 5% group O red blood cells was added. The plate was then covered and incubated for 45 minutes at 37°C. The ASO titre is the reciprocal of the highest serum dilution showing no haemolysis (cells form a button on the bottom of the well). ASO titres < 200 were considered normal.

The ADB titre was determined using the Streptonase B kit from Wampole (Carter Wallace, Sydney, Australia). A 1:60 and 1:85 dilution of patient serum with buffer was prepared. To a microtitre plate, 25 uL of buffer was pipetted to wells B to H, in columns 1 and 2. To column 1, wells A and B, 25 uL of 1:60 dilution was added. To column 2, wells A and B, 25 uL of 1:85 dilution was added. The samples were serially diluted in 25 uL aliquots from B to H, discarding the final 25 uL. To all wells, 25 uL of working enzyme solution was added. The microtitre plate was incubated for 20 minutes at 37°C. Fifty microlitres of substrate was added to all wells. After incubation for 4 hours at 37°C the ADB titres were determined. A blue or blue-violet colour indicates lack of enzymatic activity (ie:enzyme inhibition by the antibody and therefore a positive result). A pink or pinkish-violet colour is indicative of enzyme activity (ie: lack of antibody inhibition). The reciprocal of the highest dilution of serum showing inhibition (blue or bluish-violet colour) is the ADB titre of the serum. Titres < 170 for ADB were considered to be normal.

RF determinations were made using the Beckman Array Protein Analyser (Beckman, Australia). This instrument quantitatively determines human RF by rate nephelometry. The lower level for RF determination by this instrument is 30 IU/mL. The nephelometric technique has been shown to be suitable for routine use and has slightly higher levels of sensitivity than the traditional methods of sheep cell agglutination and latex agglutination for rheumatic disorders other than rheumatoid arthritis [60].

BFV and Flavivirus antibody determinations were kindly performed at the State Health Laboratory in Brisbane, Queensland. An indirect EIA prepared "in-house" was used to determine the presence of IgG and IgM antibody to BFV. Equivocal results for IgM antibody were resolved by sucrose density gradient ultracentrifugation and repeat testing in an HI assay (personal communication - Debbie Phillips, State Health Laboratory). Flavivirus antibody determination was made using HI and an indirect "in-house" EIA for flavivirus IgG and IgM. Positive IgM results were confirmed by sucrose density gradient ultracentrifugation and HI using individual flavivirus antigens (MVE, Alfuy, Kunjin, dengue 1, dengue 2, dengue 3, dengue 4, Edge Hill, Kokobera and Stratford).

Lyme borrelosis serology was performed in the laboratory by screening sera in a fluorescent treponemal absorption assay (FTA) (Diagast Laboratories, Medical Dynamics, Sydney, Australia). In this assay, patient sera is diluted in sorbent, mixed and incubated at room temperature for five minutes. The diluted sera and controls are added to wells in the slide and are incubated for 30 minutes at 37°C. After washing, conjugate is added and the slide is incubated for 30 minutes at 37°C. The slide is washed and then coverslipped. Patients with lyme antibodies react in a FTA test [38] but do not react in the *Treponema palladium* haemagglutination assay (TPHA). The TPHA was used to exclude

patients with prior exposure to syphilis. Because the FTA was being used as a screening test, any doubtful or positive test results were referred to the State Health Laboratory in Brisbane for confirmation. This laboratory uses a standard IFA incorporating an American isolate of *B. burgdorferi* as antigen. Antibody titres < 1:64 were considered negative.

A Titertek Microplate Washer S 8/12 (ICN Biomedicals Australasia) was used for all EIA assays. Plates were read on a Titertek Multiscan MCC (ICN Biomedicals Australasia). A Zeiss Axioskop 20 (Carl Zeiss Australia) microscope was used for microscopy. The fluorescent FITC filter set, 09, was comprised a broad - band interference excitation filter BP 450 - 490, barrier filter 4P420 and chromatic beamsplitter FT510. Chi square statistical analysis using 95% confidence limits for the data, was made using the Epi Info word processing, database, and statistics program for epidemiology on microcomputers (Communicable Diseases Section, Canberra).

CHAPTER 3

RESULTS

Ross River Virus Serology

Of the 524 serum samples included in this study, 204 or 38.9%, had evidence of exposure to RRV as indicated by the presence of specific IgG and/or IgM antibodies. A total of 103 of 213 male sera (48%) and 101 of 311 female sera (32%) comprised this figure. There was a positive association between male sex and the presence of RRV antibodies which achieved statistical significance $(P < 0.05)$. The youngest patient with antibodies to RRV was a ten year old female with IgG and IgM antibodies who was noted to have arthralgia. The oldest patient with both IgG and IgM antibodies was a 76 year old female who had no clinical history provided on the request form. The prevalence of RRV antibody was found to increase with age and this is shown in Table 1. The rate of increase calculated from a line of best fit computed by linear regression was 0.85% of the population per annum. The seasonal distribution of patient sera with detectable IgM to RRV (taken as presumptive evidence of acute infection) for the years 1991 and 1992 combined is shown in Figure 1. Data from routine laboratory work is included in this figure to increase the data pool. The numbers of patients in which RRV IgM was detected was highest in the hotter months which also typically have higher rainfall totals recorded.

A summary of the RRV antibody profiles is shown in Table 2. Samples with RRV IgM antibody only present, were found to be repeatedly positive in the PanBio EIA.

A review of the patient request forms looking specifically at the patient clinical history revealed that of the 60 patients with detectable IgM, 21 had no clinical history provided. In the remaining patients, 21 had arthralgia/joint

Table 1. Prevalence of anti-Ross River virus IgG/IgM in Central Queensland residents requiring laboratory diagnosis of PUO.

AGE	NUMBER OF PATIENTS	
(YEARS*)	anti-RRV IgG/IgM	NO DETECTABLE RRV Ab.
1 - 10	1 (10%)	9
11 - 20	7 (14%)	44
21 - 30	30 (31%)	67
31 - 40	42 (34%)	80
41 - 50	57 (48%)	62
51 - 60	34 (52%)	31
61 - 70	17 (53%)	15
71 - 80	14 (74%)	5
81 - 90	1 (20%)	4

* Four patients had no age given on the request form and their results are not included in this table.

Figure 1. Seasonal distribution of detection of anti-RRV IgM antibody in sera from patients requesting PUO serology. The results are for the years 1991 and 1992 and includes samples from both the confines of this project and from those from routine laboratory work in order to increase the data pool.
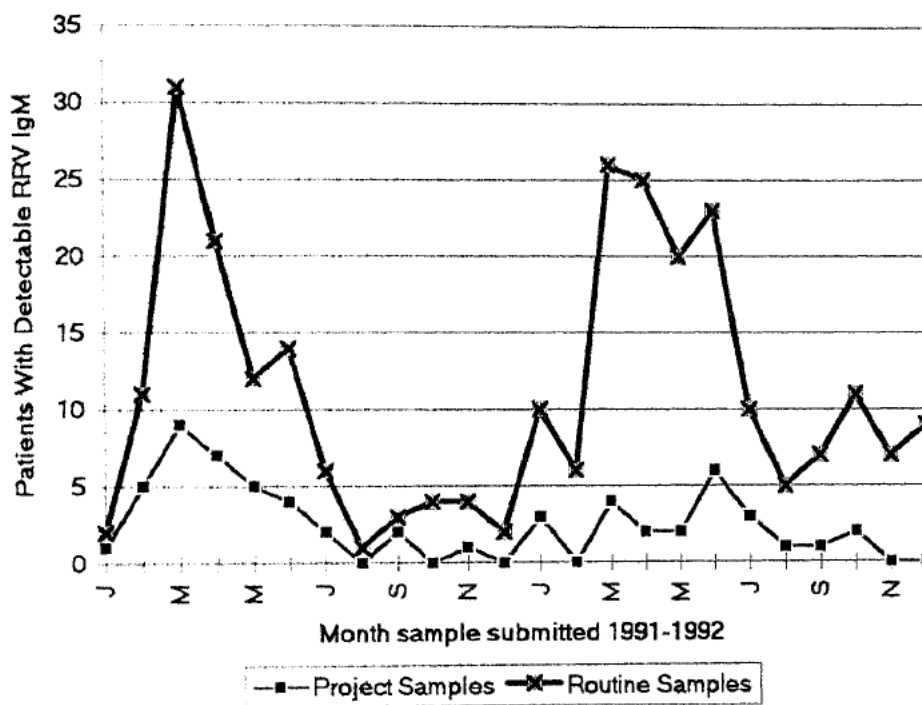
Table 2. A summary of the antibody profiles for patient sera collected in 1991 and 1992. All antibody determinations were made by EIA.

No. of Patients	Antibody Profiles	
	IgG	IgM
320	-	-
144	+	-
42	+	+
18	-	+

pain, 9 had myalgia/muscular pain, 6 had a rash noted and 3 patients were noted to have a fever. Two patients were specifically noted to have "no rash" but both patients were noted to have arthralgia. One patient was also noted to have nausea and headaches in addition to arthralgia involving wrists, ankles and shoulders.

One hundred and forty-four patients had RRV IgG with no RRV IgM antibody detected. Whilst 69 patients provided no clinical history, 34 had a history of arthralgia, 13 patients had myalgia, 10 patients had fever, 9 patients had lethargy, 7 patients had malaise and 3 patients had a rash noted. Other symptoms listed included nausea, chills, weight loss, cough, viral illness, PUO, headaches, swelling in the joints, meningism, oedema, upper respiratory tract infection and lymphadenopathy.

Three hundred and twenty patients had no detectable antibodies to RRV. Of these, 140 patients had no clinical history provided on the request form. Arthralgia or joint pain was noted in 91 patients. In the remaining patients, 35 patients were noted to have myalgia, 32 patients had fatigue/lethargy, 16 patients had fever, 11 patients had a viral illness noted, 9 patients had a rash, 8 patients had PUO, 7 patients had headaches and 4 patients were noted to have lymphadenopathy. Some patients had a combination of the above clinical symptoms.

A wide variety of other pathology tests were requested in conjunction with the RRV serology requests. These request numbers are listed as follows:

full blood count	423
erythrocyte sedimentation rate	355
rheumatoid factor determination	139
Epstein Barr virus serology	84

liver function tests	78
urea and electrolytes analysis	75
infectious mononucleosis serology	52
Flavivirus/Dengue virus screen	48
autoantibody screen	44
Cytomegalovirus serology	40
thyroid function tests	33
blood glucose analysis	33
Barmah Forest virus serology	28
renal function tests	25
Streptococcal serology	25
Q fever serology	23
mid-stream urine analysis	21
Hepatitis serology	14
Brucella serology	14
Toxoplasma serology	11
Leptospirosis serology	10
Lyme Borrelosis serology	7
Rickettsial serology	5
Human Immunodeficiency virus serology	5
Mycoplasma serology	5
blood cultures	1

The full blood count results for patients with detectable Ross River virus IgM antibody were all normal with no atypical lymphocytes reported from the blood film. The erythrocyte sedimentation rate values ranged from 3 to 42 with a mean of 14.6 (normal range < 10 mm/hour). Additional pathology tests were not requested on 49 patients.

Leptospirosis Serology

For the period 1991/1992, ten of the survey samples had requests for leptospirosis serology in addition to RRV serology. This group was comprised of 5 females aged 2 to 55 years of age and 5 males aged 22 to 59 years of age. The clinical history provided on these request forms included lethargy, arthralgia, fever, chills, myalgia, lymphadenopathy, rash and an association with cattle. One of these patients had a positive screening test with the MSAT which was subsequently determined to be negative by MAT at the reference laboratory. In addition, 15 other samples in the survey were positive by MSAT but were negative by MAT.

One of the patients with a request for leptospirosis serology had IgG and IgM antibody to antigen of the spotted fever group of rickettsia, suggesting recent infection. This patient was a 55 year old female presenting with fever, rash, chills and joint swelling. The patient did not have a specific request for rickettsial serology.

Flavivirus Serology

One hundred and three of the 524 (19.7%) patient samples had evidence of exposure to flavivirus antigen as demonstrated by the presence of IgG antibody. There was a borderline statistical association between sex and exposure to flavivirus antigen (P = 0.5). The youngest patient with detectable IgG antibody was a 12 year old male with arthralgia. The oldest patient was an 86 year old female, also with arthralgia. A 52 year old female presenting with fever and arthralgia had IgG and IgM antibody present to both Kunjin and Alfuy viruses. The HI titre for this patient was 1:1280. The patient serum was

received in September 1991, a month in which there was no recorded rainfall. The previous months of July and August had rainfalls of 26.4 and 7.4 mm, respectively. These months are also typically the coldest months of the year for the Mackay district. The patient had no recent travel history and had no other abnormal laboratory test results.

The age distribution of patients with detectable flavivirus antibody as measured by EIA is shown in Table 3. Evidence of exposure to both RRV and flavivirus antigen was detected in 69 patients, whilst 34 patients had flavivirus antibodies but no RRV antibodies detected. These results show a statistical association between exposure to RRV and flavivirus antigen (P < 0.05).

Forty eight requests for flavivirus or dengue serology were received of which 11 patients had IgG antibody present. Two of these patients had RRV IgG and IgM antibody present. One patient had IgG and IgM antibody to the spotted fever group rickettsial antigen.

Lyme Borrelosis Serology

A positive FTA result in nine patients was considered as presumptively evidence for lyme borrelosis. All of these patients had a negative TPHA test. Seven patient sera (including one patient who presented twice) were found to have IgG antibodies to *B. burgdorferi* in the IFA test performed at the reference laboratory. These patients included 4 females and 2 males with ages ranging from 34 to 67. Titres were 1:64 for 2 patients, 1:128 for 3 patients and 1:512 for 1 patient. IgM antibodies were not detected in any sera. One patient presented a second time one month later with persistent arthralgia and myalgia without any change in IgG titre (1:64). All other serological tests were negative on both occasions excepting a rise in ADB titre from 1:60 to 1:240. The patient with the

Table 3. Prevalence of anti-Flavivirus IgG/IgM in Central Queensland residents requiring laboratory diagnosis of PUO.

AGE	NUMBER OF PATIENTS	
(YEARS[*])	anti-FLAVIVIRUS IgG/IgM	NO DETECTABLE FLAVIVIRUS Ab.
1 - 10	0 (0%)	10
11 - 20	3 (6%)	48
21 - 30	5 (5%)	92
31 - 40	8 (7%)	114
41 - 50	22 (18%)	97
51 - 60	27 (42%)	38
61 - 70	20 (63%)	12
71 - 80	15 (79%)	4
81 - 90	3 (60%)	2

[*] Four patients had no age given on the request form and their results are not included in this table.

antibody titre of 1:512 was febrile with arthralgia and myalgia and had RRV IgG and IgM antibodies present.

Two patients had no clinical history supplied. All four remaining patients had arthralgia. Myalgia was also noted in two patients. None of the patients with antibodies against *B. burgdorferi* had requests for this serology, however seven other requests were received. Sera from two of these patients contained RRV IgG and IgM antibodies and another had IgM antibodies to the spotted fever group of rickettsia. Sera from two patients contained RRV IgG antibody while in the sera from the remaining two patients, no positive serology results were recorded. The clinical history for this group of patients included arthralgia in four patients, fever in two and myalgia in one patient with one patient having no clinical history provided on the request form.

Barmah Forest Virus Serology

Anti-BFV IgG antibodies were detected in 42 (8%) patients. Three of these patients also had IgM antibodies. The first positive serum was received in May 1991 from a 57 year old female. This patient had become ill one month earlier with severe headaches and fever. Her left foot had severe pain to the extent that she could barely walk upon it. The right foot also developed pain but not to the same extent. This patient also had IgG and IgM antibodies to RRV and IgG antibodies to flavivirus antigen. The reference laboratory found anti-BFV and anti-RRV antibodies were of similar titres (HI titre = 1:1280). This sample and a subsequent sample remained positive to both RRV and BFV IgM antibodies after ultracentrifugation. The second positive specimen was received in April 1992 from a 40 year old male with arthralgia involving wrists, ankles and shoulders. He also had lethargy, nausea and headaches and was specifically noted to have no rash. This patient also had RRV IgG and IgM antibodies and

flavivirus IgG antibodies. The third positive specimen was from a 40 year old female with shoulder pain and was also received in April 1992. Her RRV and flavivirus serology was negative. None of these patients had specific requests for BFV serology.

The prevalence of BFV antibody as determined by EIA is shown in Table 4. The rate of increase calculated from a line of best fit computed by linear regression was 0.27% of the population per annum. There was no statistical association between sex and seropositivity (16 males, 26 females; $P = 0.73$). There was however a positive statistical association between BFV exposure and both RRV ($P < 0.05$) and flavivirus exposure ($P < 0.05$). Twenty nine of 42 BFV positive samples also had RRV antibodies whilst 20 of 42 also had flavivirus antibodies.

Of the 524 samples included in the survey, 28 (5%) of the request forms included a request for BFV serology. Only one of these patients had detectable antibody. The clinical history for patients with antibodies to BFV as indicated on the request forms included arthralgia in 13 patients, myalgia in 6 patients, a rash in 4 patients, headaches in 3 patients, nausea in 2 patients and fever was noted in 2 patients. Some patients had a combination of the above clinical symptoms.

Brucella Serology

A total of 14 requests (10 male patients and 4 female patients) were received for brucella serology in conjunction with RRV serology. One of these, a 45 year old male pig shooter with headaches and night fevers had titres of 1:5120 to *B. abortus* antigen and 1:2560 to *B. melitensis* antigen. Blood cultures on this patient were requested and three of six became positive for *B. suis* biotype

Table 4. Prevalence of anti-Barmah Forest virus IgG/IgM in Central Queensland residents requiring laboratory diagnosis of PUO.

AGE	NUMBER OF PATIENTS	
(YEARS*)	anti-BFV IgG/IgM	NO DETECTABLE BFV Ab.
1 - 10	0 (0%)	10
11 - 20	1 (2%)	50
21 - 30	1 (1%)	96
31 - 40	12 (10%)	110
41 - 50	11 (19%)	108
51 - 60	11 (17%)	54
61 - 70	2 (6%)	30
71 - 80	4 (21%)	15
81 - 90	0 (0%)	5

* Four patients had no age given on the request form and their results are not included in this table.

one within one week. The remaining blood cultures remained negative until discarded after one month. Two other patients also had detectable antibodies. A 42 year old female with arthralgia and lethargy had initial titres of 1:80 to *B. melitensis* antigen and 1:160 to *B. abortus* antigen. A second serum sample two weeks later was negative in both tests (< 1:80). The second patient, a 21 year old female with pain in her hands and feet had titres of 1:320 to *B. abortus* antigen and 1:160 to *B. melitensis* antigen. A second specimen one week later had titres of 1:160 to *B. abortus* antigen and 1:80 to *B. melitensis* antigen. This patient was also positive for RRV IgG and IgM antibodies.

The clinical history supplied for patients with requests for brucella serology included arthralgia in three patients, fever in two patients, PUO in two patients, malaise in one patient and lethargy in one patient. One patient was noted to be a butcher with arthralgia for one week and was positive for RRV specific IgM. Two other patients (father and son), worked with cattle and had fever but were negative to all serological tests performed on initial testing and again two weeks later.

Rickettsial Serology

Eighteen of the 524 (3.4%) patients had detectable antibodies to the spotted fever rickettsial organism - *R. conorii*. These patients included 11 females and 7 males (no statistical association between sex and exposure : P = 0.9), with ages ranging from 20 to 73 years of age. Only one of these patients had a request for rickettsial serology and was found to be IgG positive and IgM negative. A further four requests were received and these patients were negative for all other serological assays performed in this study. The clinical history supplied for these five patients included arthralgia in four of the patients, a rash in three patients, fever in two patients, and myalgia, malaise or PUO in one patient each.

The clinical history supplied for the 18 patients with antibodies to *R. conorii* included arthralgia in five patients, fever in four patients, rash in two patients, generalised pain in two patients, and vomiting, myalgia, swelling, chills and weight loss were noted in one patient each. Some patients had a combination of the above clinical symptoms. There were seven patients with no clinical history supplied.

Four patients were found to be IgG antibody negative and IgM antibody positive. Two of these patients had no clinical history supplied. The first patient was negative for all serological tests performed in this study and the second patient had an ASO titre of 1:200 as the only notable result. The third patient in this group had a clinical history of fever and pain and was negative to all other tests. The final patient had myalgia, pain and vomiting and was RRV IgG and IgM antibody positive and had an ASO titre of 1:400.

Four patients were positive for both IgG and IgM antibodies. These patients included one patient who was also RRV IgG antibody positive, one patient who was also RRV IgM antibody positive and one patient who was both RRV and flavivirus IgG antibody positive. All four patients had arthralgia with two patients also reporting a rash.

Ten patients had IgG antibodies but no IgM antibodies to the *R. conorii* antigen. One of these patients was also RRV IgG and IgM antibody positive. A second patient had an elevated ASO titre of 1:400 whilst a third patient had an ASO titre of 1:200 and ADB titre of 1:240. Three patients were also found to have RRV IgG antibodies and the remaining four patients had flavivirus IgG antibodies.

An interesting feature noted was the general lowering of serum

electrolyte levels in some of the patients. In one extreme case, the serum sodium level was 117 mM/L (normal range:135 - 145, critical:115 - 116) and the chloride level was 79 mM/L (normal:98 - 108, critical: 70). This is probably due to the tropism of the rickettsial organisms to invade the capillary vascular epithelium, causing damage and resulting in volume depletion of the circulatory system [51]. In a patient from outside the confines of the study showing a seroconversion in the Weil - Felix and IFA tests, hepatic dysfunction occurred which initially suggested to the general practitioner, an acute infection with either of the hepatitis A or B viruses.

Streptococcal Serology

A total of 25 requests were received for streptococcal serology in addition to the request for RRV serology, with 142 of the 524 patient sera (27.1%) having raised ASO and/or ADB titres. Sera were considered abnormal if the ASO titre was ≥200 or the ADB titre was ≥170. Eighteen of these patients were positive for RRV IgM antibody and three patients had rickettsial IgM antibody present. Forty seven patients had evidence of exposure to RRV, flavivirus, rickettsia, BFV or *B. burgdorferi* as evidenced by detectable IgG antibodies. The remaining 74 patients with abnormal streptococcal serology results had no positive results in any of the other serology tests performed. Just five of these patients had requests for streptococcal serology.

No clinical history was provided for 29 of the patients with abnormal streptococcal serology results. In the remaining patients, arthralgia was noted to be present in 23 patients. Myalgia and pain were noted in seven patients each. Lethargy and fatigue were noted in five patients, headaches in four patients, a PUO or viral illness in three patients, rash, malaise or lymphadenopathy in two patients and fever was present in one patient. Some patients had a combination

of the above clinical symptoms.

Rheumatoid Factor

Rheumatoid factor was >30 IU/mL in 24 patients. A total of 139 requests were received for RF determination and 10 of these had RF >30 IU/mL. The youngest patient with detectable RF was a 17 year old female with polyarthritis whilst the oldest was a 78 year old female. This patient had no clinical history provided. Other symptoms noted in patients with detectable RF included arthralgia in eight patients, fever in two patients, PUO or malaise in two patients and myalgia, rash or viral illness in one patient each. Some patients had a combination of the above symptoms.

RRV IgM antibody was detected in five patients and rickettsial IgM antibody was detected in one patient with elevated RF titres. IgG antibodies to flavivirus, BFV or RRV was present in eight patients with elevated RF. Two patients had elevated streptococcal titres.

CHAPTER 4

DISCUSSION

The Mackay district has a peak period of RRV infections from January to June which coincides with seasonally higher rainfalls and temperatures. The association between males and the presence of RRV antibodies in this study is in contrast to previous studies in which the prevalence of antibody in both sexes has been approximately the same [8, 9]. The number of female patients presenting to their doctor with symptoms suggesting a RRV etiology however was 1.46 times as many as male patients which would influence the above results. This result may reflect sex related differences as males tend not to seek medical advice readily. The ratio of female patients to male patients with IgM antibody to RRV (presumptive evidence of acute infection) is similar to that reported by Aaskov et al. in 1981 [9]. In this study conducted in the Central Queensland region, 43 females and 24 males developed symptoms associated with acute RRV infection (ratio = 1.79:1). For the study reported here, 38 females and 22 males had IgM antibody to RRV (ratio = 1.73:1). In the Fiji epidemic, the female to male ratio reported was 1.71:1 [8].

An analysis of antibody distribution by age group (Table 1), suggests that by the fifth decade of life, approximately 50% of Central Queensland residents presenting for evaluation of PUO, have been exposed to RRV as evidenced by the presence of antibodies detectable by EIA. The infection rate was calculated to be 0.85% of the population per annum. This compares to the previous studies of Phillips et al of 0.59% per annum [44] and Aaskov et al of 1.4% per annum [9]. The largest group of patients presenting with clinical symptoms suggesting a RRV etiology is those aged between 21 and 60. Only 10% of patients in the study were under 20 years of age suggesting that symptomatic infection is not as apparent in this age group as it is in adults. This result supports observations

from other studies [8, 9].

A disturbing feature noted is that 43.9% (230 of 524) of the request forms received by the laboratory had no clinical history accompanying the patient serum despite there being space allocated on the pathology request form. It is difficult for the laboratory to assist the doctor in the interpretation of the serology results without clinical history. This places a greater emphasis on the doctor to fully understand the potential problems associated with arbovirus serology results, especially those based on single serum samples. The results of serological assays should be interpreted in conjunction with the clinical symptoms and the onset date of the symptoms.

From the clinical history supplied, the predominant feature in patient's with detectable RRV IgM was arthralgia and joint pain. Other symptoms noted included myalgia, rash and fever, all of which are consistent with previous studies of RRV symptomology [11]. The patients with evidence of previous infection (IgG antibody positive and IgM antibody negative) also had similar symptoms noted, as did the patients with no serological evidence of exposure to RRV. These results show an understanding by the doctors of the symptoms associated with RRV infection. These symptoms however can easily be transposed to infection with other agents associated with pyrexia and polyarthralgia which may be overlooked in the differential diagnosis of PUO.

An examination of the requesting practices used by the doctors when ordering RRV serology revealed that the full blood count, erythrocyte sedimentation rate and RF determinations were by far the most commonly ordered additional tests. The results of the erythrocyte sedimentation rate and RF can be useful in differentiating the etiology of infection in that both are generally normal in acute RRV infection [10, 11]. The full blood count typically

shows a normal profile during RRV infection but may occasionally show atypical lymphocytes [10].

Five patients with Ross River virus IgM antibody had RF detected. Three patients had RF values from 30.4 to 31.1 IU/mL which is just above the lower limit of detection of 30 IU/mL. A 59 year old male had a RF of 254 IU/mL and a 65 year old male had a RF of 359 IU/mL. The measurement of IgM RF is the most useful serological test for the diagnosis of rheumatoid arthritis [58], however high titres may also be found in some chronic infections and autoimmune diseases. As with other serological tests, results must be interpreted in light of the clinical presentations. The presence of RF in patients presenting to their doctor for investigation of "acute" symptoms is probably a misleading result with a low positive predictive value as evidence of rheumatoid arthritis [57]. The observation that RF was detected in 5 of 60 patients with Ross River virus IgM is unusual. In a series of patients studied by Oseni *et al.*, RF was only detected in 2 of approximately 1000 patients [61]. In another series of patients from Victoria, only 1 of 125 patients had detectable RF [10. These results may represent experimental differences as the minimum detection limits for RF were not stated in either of these studies. Patients with both RF and RRV IgM antibody should be further evaluated on clinical grounds and by following the clinical course of the disease.

No cases of leptospirosis were detected in any of the patients for this period of study. Routine work through the laboratory only detected one patient with an acute leptospiral infection for the same period. The low numbers of requests for leptospirosis serology may suggest a feeling of a low prevalence of this infectious disease in the Mackay district by the general practitioners. During the period 1991 - 1992, 87 cases of leptospirosis were notified from throughout Queensland to the Department of Health (based on weekly

notification reports).

The MSAT system from Diagnostics Pasteur was chosen in this laboratory because it is suited to low test numbers, is easy to perform and gives good results. A recent evaluation of the test found it to be 100% specific with a negative predictive value of 79% [62]. The modification used in this laboratory of reading the agglutination with low power darkfield microscopy would appear to have reduced the test specificity as evidenced by the number of positives in the assay that were not confirmed by the reference laboratory. The false positive reactions were not considered to be problematic as the assay was used as a screening assay with doubtful or positive results being retested by MAT at the reference laboratory.

The low level of flavivirus antibodies detected indicates a low level of flavivirus activity in the Mackay district. The Mackay district has missed the dengue epidemics of recent times despite the vector *A. aegypti* being found in the district [63]. Flavivirus antibodies are not cross protective and thus this low prevalence of antibody also indicates that any of the four dengue serotypes could easily become established if introduced. The Mackay district would seem to be very vulnerable to this type of epidemic. The higher level of antibody prevalence in the older age groups (from the fifth decade of life on) suggests the length of time that has elapsed since previous significant flavivirus activity in the district. These results are in contrast to those for Ross River virus which appears to be endemic to the Mackay district. One patient's results did return a presumptive diagnosis of acute flavivirus infection based on IgM detection to Kunjin and Alfuy viruses. The patient's symptoms of fever and arthralgia supported the diagnosis but the onset date of symptoms were not noted.

The positive statistical association between exposure to RRV and

flavivirus antigens probably reflects continued exposure to mosquitoes. This is also the case with exposure to BFV. The prevalence of BFV antibodies was 8%. The calculated seroconversion rate for BFV was 0.27% per annum which compares with that of Phillips *et al* of 0.23% per annum [44]. Three patients had detectable IgM antibody. Two of these patients also had Ross River virus IgG and IgM antibodies which raises the question of possible dual infections, cross reactions in the serological assays or an anamnestic response to some other infectious agent. BFV has been isolated from vectors associated with RRV and it is possible that the two viruses could be circulating simultaneously in the same mosquito population. Another feature supporting the existence of dual infections is that alphavirus IgM is generally considered to have low levels of cross reactivity [2]. Of the 11 patients with RRV IgM and BFV IgG antibodies, only 2 patients had BFV IgM detected. If there were specificity problems associated with the IgM assays for the alphaviruses, we would expect to see more patients presenting with detectable IgM to both RRV and BFV. It is not possible to comment on all sera with RRV IgM antibody present for the presence of BFV IgM antibody as the reference laboratory only reported specific BFV IgM serology results when BFV IgG antibodies were detected.

The patients with detectable BFV IgM antibody presented in the months of March, April and May. This is also the period for peak RRV activity and may be expected considering the known mosquito vectors associated with BFV transmission.

The small number of requests for lyme borrelosis serology indicates either a low index of suspicion for infection with this organism or a lack of knowledge concerning this organism or both. Doctors contacted with positive antibody results from their patients were not familiar with the organism *B. burgdorferi* and its disease spectrum. It is very difficult to determine if any of

the seven patients with positive antibody results were acutely infected with the lyme disease spirochete based on the available results. No patient had detectable IgM antibody and only three patients were negative for all other assays performed in this study. These results do not exclude the possibility of another infectious agent being responsible for the patient's symptoms. One patient presented one month after the initial visit but had no change in titre in the IFA test (1:64). This patient with myalgia and arthralgia did show a rise in titre of ADB from 1:60 to 1:240 suggesting a streptococcal infection.

Cross reactivity in serological assays for lyme borrelosis limit their usefulness as a diagnostic tool. False positives may occur in patients with tick-borne relapsing fever, louse-borne relapsing fever, syphilis and Rocky Mountain spotted fever [38]. None of the patients within the confines of the study reacted with both lyme antigen and the spotted fever group rickettsia antigen. The laboratory has had experience with two other patients with demonstrated seroconversion in the *R. conorii* IFA assay and rising titres in the Weil-felix agglutination assay who also had positive lyme antibody results. One of the patient's serum specimens was referred to the Centres for Disease Control (CDC), Fort Collins in Colorado, USA, for testing in their serological assay. The patients optical density ratio in their EIA system was 0.175 which is below the CDC criteria of 0.2 for positive specimens. These results suggest a false positive result, possibly due to cross reacting antibody from the rickettsial infection.

To date, there has not been an isolate of *B. burgdorferi* in Australia from patients without an overseas travel history, or from various Australian tick or animal species. Recently an isolate identified as *B. burgdorferi* was isolated from a patient in Queensland with a long term history of lyme borrelosis previously diagnosed in the USA [64]. The geographic source of this isolate may be elucidated upon completion of further characterization of the organism. Until

there is a proven Australian isolate, the diagnosis of lyme disease in Australia will remain an uncommon occurrence.

Brucellosis is a disease which should always be considered as part of a differential diagnosis of PUO in rural workers. There has been an increase in the number of cases of brucellosis diagnosed in Queensland in recent years, the reasons for which may lie in the increasing market for feral pig meat destined for overseas consumption [65, 66]. *B. suis* culture and/or serology positive feral pigs have been detected throughout much of central and western Queensland [66]. The evidence suggests that *B. suis* is responsible for the majority of cases of brucellosis in Queensland [65, 66]. Most of the requests for brucella serology (9 of 14) were also accompanied by requests for Q fever serology indicating an association by the doctors for this disease and the cattle industry despite the fact that the Queensland cattle population was declared free from *B. abortus* on January 1, 1989. *B. abortus* may still occur as evidenced by the isolation of this organism from patients in each year since this announcement [65, 66].

The patient diagnosed with brucellosis in this study had a typical presentation, being a pig shooter with night fevers and headaches. All other serology for this patient was negative and the diagnosis was confirmed by isolation of *B. suis* biotype one from blood cultures. The two other patients with positive SAT titres probably represent non-specific reactions as diagnostic titres and changes in these titres could not be demonstrated. Cross reactions with other organisms do occur [46] and higher SAT titres may be found in people occupationally exposed to infected animals. Diagnosis of infection should be confirmed by isolation of the organism, by the demonstration of rising titres between appropriately collected serum specimens or the detection of specific IgM antibody.

Failure to include the possibility of rickettsial infections in patients presenting with a PUO may prolong patient morbidity as treatment is readily available even though many patients symptoms resolve without medical intervention. Eighteen patients had antibodies to the spotted fever group antigen used in the IFA test. None of these patients had any recorded tick bite noted on the request form and presumably were not questioned on this point as this may have indicated a possible rickettsial etiology. It is well recognised however that many patients do not recall contact with ticks [53]. The patient who had positive IgM antibody results for both RRV and QTT provides a diagnostic dilemma. Further serological investigations to determine rising or falling titres would be required to elucidate the identity of the infectious agent. A detailed investigation of the patient's clinical history may also prove useful. This is another example of the problems which may be associated with single serum sample testing.

Antibody as detected by IFA of the IgM class appears from six to ten days after onset of symptoms and persists for about ten weeks whilst IgG antibody appears after about two to three weeks and persists for about one year [51]. These figures place the time of infection of 10 of the patients between 3 and 12 months prior to the laboratory receiving the serum sample and so it is unlikely that their current symptoms are related to the rickettsial infection. Another point to consider is that the IFA test used in the laboratory uses a surrogate antigen for the presumptive diagnosis of *R. australis* infection. It is probable that use of *R. australis* antigen would have given higher antibody titres and so additional patient sera may have reacted in the IFA test. Clinical history for patients with IgM antibody present included arthralgia, rash and fever, symptoms identical to those seen in patients with RRV infection. These diverse clinical presentations outline the problems faced by the doctor when encountering a patient presenting with PUO.

The results from the streptococcal antibody testing were most surprising. These showed that 27% of patients had raised ASO or ADB titres. Raised titres were found in 18 of 60 positive patient samples for RRV IgM antibody and 3 of 8 patients with IgM antibody to the spotted fever group rickettsia antigen. These results are difficult to interpret because of the inherit problems with single sample testing. There would undoubtedly be overlap of "abnormal" results considering that persistence of IgM antibody to RRV can occur [2, 13] and that ASO and ADB titres may not return to normal for many months or years [56]. However 14% (74/524) of results were abnormal in the absence of any other serological abnormalities. In addition, another 9% (47/524) of the abnormal streptococcal serology results were associated with the presence of IgG antibodies to other agents tested which indicates infection at some time in the past. In these patients, clinical history included arthralgia, myalgia, lethargy, rash, pain, malaise, lymphadenopathy and fever, all of which may be associated with the more "common" agents associated with PUO. These results would suggest that medical intervention could reduce patient morbidity in a significant proportion of the population presenting with a PUO.

CHAPTER 5

CONCLUSIONS

The primary aim of the continued monitoring of pathogenic organisms is to aid in patient management and to reduce morbidity and mortality. The changing global climatic conditions necessitate continued surveillance in order to detect the introduction and proliferation of tropical diseases. The economic consequences of epidemics of infectious disease are difficult to evaluate but the costs are shared by the entire community .

The numbers of patients appearing to have markers of recently acquired infection (IgM antibody) to more than one etiologic agent is a cause for concern. These results highlight the need for accurate clinical history and the necessity for appropriately collected serum samples in order to make a firm diagnosis in many patients. Acute phase sera should be collected within seven days of the onset of symptoms. A presumptive diagnosis of infection can be made on this sample on the basis of the presence of IgM antibody with or without the presence of IgG antibody or antibody detected by CF, HI or NEUT assays. To confirm the diagnosis, a demonstrable fourfold rise in antibody titre must be shown between this sample and a convalescent phase serum sample collected within 15 to 28 days of the onset of symptoms. For patients who do not present in the acute phase of infection, a fourfold decline in antibody titre may be necessary to confirm the diagnosis.

A problem faced by most laboratories is the lack of appropriate clinical information provided by the referring doctor. This makes it very difficult for the laboratory staff to assist with the interpretation of results. The problem faced by doctors is the time taken to inform the laboratory of the relevant clinical information and the reluctance to have the patient pay for two or more

pathology tests which can amount to a considerable financial burden. There is also a perception that a single sample with IgM antibody is all that is required to make a diagnosis. An additional problem faced by the laboratory is the selection of suitable testing protocols and testing methodologies. It is known that different diagnostic products have obvious differences in sensitivity and specificity. There is an need for the laboratory staff to select the most appropriate testing facilities for their population of patients. A product that performs well in an area with a high prevalence of a particular disease may be very erroneous in a population with a low prevalence of that same disease. This again emphases the need to know the prevalence of infectious agents in the community.

The results from this study show that Ross River virus is endemic in Central Queensland. Current vector control measures appear to be ineffective in reducing the incidence of mosquito-borne diseases. Perhaps some of the costs involved in vector control should be redirected towards vaccine research which may in the future have economic benefits for the entire community. An interesting result is the low level of flavivirus antibody which suggests the possibility of future epidemics of flavivirus disease. With the recent epidemics of dengue fever occurring in Townsville and Cairns and given the relatively short distances between these population centres, it would seem inevitable that dengue infections will occur in the Mackay district. At the time of writing, six cases of dengue serotype two infection (presumptive infection as evidenced by the presence of IgM antibody) have been reported in the Mackay district in the first half of 1993. Some of these patients have no history of recent travel.

Leptospirosis and brucellosis, although uncommon in the Mackay district, should not be forgotten in the differential diagnosis of PUO. The incidence of brucellosis in Queensland appears to be increasing [65, 66], whilst cases of

leptospirosis continue to be reported each year. Lyme borrelosis as recognised in other parts of the world may not yet exist in Central Queensland. The detection of low levels of antibodies in patients probably represents nonspecific reactions. A feature borne out by the results is the apparent under - diagnosis of infections due to rickettsia and streptococci.

In the first description of Ross River virus disease by Nimmo, he writes of "a disease which I cannot nail down as any known epidemic, nor cast into the practitioner's "dustbin" of diagnosis and name "influenza"" [3]. The results from this study suggest that beside the "influenza dustbin", there may sit a "Ross River dustbin".

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