الاستجابة المناعية الخلطية في التهاب الكبد الفيروسي المزمن, الحمى المتموجة وداء السكري

أطروحة مقدمة الى مجلس كلية العلوم-جامعة بابل وهي جزء من متطلبات نيل درجة الدكتوراه- **فلسفة في** علوم الحياة/ **الأحياء** المجهرية

من قبل

آمال مرزة سن

1424 هـ **2003**م

بسم الله الرحمن الرحيم

((سنريهم ءايتِنا في الآفاق وفي أنفسِهم حتى يتبيّنَ لهُم أنهُ الحق أو لمْ يكفِ بِربِّكَ أنهُ على كل شيءٍ شهيدٌ))

صدق الله العظيم

(فُصِّلَت53)

5. Discussion:

5.1. C- reactive protein (CRP):

The separation of the CRP from human serous fluids was first described in 1947 (Mc Carty, 1947). In order to separate and identify the CRP, pleural fluid from patients with an acute infection was used, and the separation procedure was done according to Mc Carty (1947). The CRP of the pleural fluid was not associated with lipid, and this property was of importance in the success of the separation procedure (Mc Carty, 1947; Wood *et al*., 1954). In physical and chemical senses, the separated fluid was consistent with the characteristics of CRP (Tillet and Francis, 1930; Tillet *et al*, 1930; Mc Carty, 1947; Wood *et al*., 1954; Pepys, 1982). Immunological studies confirm earlier work in showing that the CRP was highly antigenic and serologically specific (table-4) (Mc Carty, 1947; Wood *et al*., 1954; Pepys, 1982). Rabbit's anti-CRP coated erythrocytes, PHAT, were found equally efficient as latex agglutination test (commercial latex agglutination test kits) in detecting CRP level in serum samples of patients and control groups (table-5).

Young and associates (1991) have made literature review to determine the value of CRP measurements in the diagnosis and management of a wide range of conditions. Measurements of CRP level are of value in six clinical situations:

1. Monitoring the response to antibiotic treatment among patients with known bacterial infections.
2. In obstetric patients with premature rupture of membranes, a rise in CRP can give early warning of intrauterine infections.
3. Differentiation between active disease and infections among patients with SLE and ulcerative colitis where the level of response to active disease has previously been established.

4. As a measure of disease activity and response to disease modifying drugs in rheumatoid arthritis.
5. Early detection of complication in post-operative patients.
6. In differentiation between infection and graft-versus-host disease in bone marrow transplant patients.

In chronic inflammations CRP levels remain abnormal (Kallio, 1997; Craig *et al*., 2002). In the present work an attempt was carried out to document CRP use in the diagnosis and/or, follow up the acute flare up of one of the chronic viral infections (CVH-B&C), one of the chronic bacterial infections (chronic brucellosis), and one of the chronic metabolic diseases (D.M). It was found that CRP level raised up during the chronic course of brucellosis, D.M, and viral hepatitis B&C compared to the normal body state (table-6). This was in agreement with Ebeling *et al*., 1999; Ford, 1999; Moran and Romero, 1999; Zhou and Fischer, 1999 and Craig *et al*., 2002. Since its discovery in 1930 by Tillet and Francis, CRP has been considered an indicator for disease activity. Its level elevated in infections and other disorders associated with tissue damage and inflammation (Kallio, 1997; Clyne and Olshaker, 1999). CRP rose up during bacterial infection (brucellosis) to higher levels than in viral infection (CVH-B&C) (table-6). This would be in agreement with Craig *et al*., 2002. CRP was synthesized in the liver by hepatocytes (Pepys, 1982). Chronic course of HBV and HCV infection might be associated with hepatocytes damage (Lindsay and Hoofnagle, 2000). CRP synthesis (figures: 2-5) increased in response to tissue damage and inflammation (Clyne and Olshaker, 1999). However, its synthesis confounded by the liver's altered synthetic capacity as a result of hepatocytes damage (Craig *et al*., 2002).

Such findings could be added to the six clinical situations that have been mentioned by Young *et al*., (1991). Hence the clinical situations in which CRP levels were of value are:

1. Monitoring the response to antibiotic treatment among patients with known bacterial infections.
2. In obstetric patients with premature rupture of membranes, a rise in CRP can give early warning of intrauterine infections.
3. Differentiation between active disease and infections among patients with systemic lupus erythematosus and ulcerative colitis where the level of response to active disease has previously been established.
4. As a measure of disease activity and response to disease modifying drugs in rheumatoid arthritis.
5. Early detection of complication in post-operative patients.
6. In differentiation between infection and graft-versus-host disease in bone marrow transplant patients.
7. CRP raised up during the chronic course of the diseases (chronic brucellosis, D.M, and CVH-B&C).
8. CRP raised up during bacterial infection (brucellosis) to higher level than in viral infection (HCV and HBV).

5.2. Immunoglobulins (Igs) class determination:

Increased Ig (IgA, IgG, and IgM) levels were observed among patients with CVH-B&C, chronic brucellosis, and in diabetics in general (tables-7 to 10) (Ritzmann and Daniels, 1975; Wright, 1982;Gazapo *et al*., 1989; Young, 1991; Musset *et al*., 1993; Nagao *et al*., 1997). Global elevations in Igs may occur in number of disorders including chronic infections/ inflammatory conditions, liver diseases, and autoimmune diseases (Brown, 1982). IgG and IgM levels increased significantly in patients with CVH-B when compared with healthy controls, whereas IgA level remained normal (EL-Shawarby *et al*., 1993; Ikeda, 1994; Zgair, 1999).

The persistence of elevated Igs in CVH-B&C patients might correlate with the chronic course of infection (EL-Shawarby *et al*., 1993; Musset *et al*., 1993; Ikeda, 1994; Nagao *et al*., 1997). The raised serum Igs level was due to increased synthesis or to impaired degradation in the liver (Wright, 1982; Kann and Gerlich, 1998). Hepatitis virus antigenemia stimulate B-cells to produce virus specific Abs. Meanwhile, increased surface expression of viral Ags on hepatocytes during infection could stimulate CD_4 T-lymphocytes to play a helper effect on B-cell to produce virus specific Abs (Musset *et al*., 1993; Zgair, 1999).

In chronic brucellosis patients, there was an increase in IgG and IgA level. However, increased IgM level could reflect an active disease process (Gazapo *et al*., 1989; Brooks *et al*., 1998; Young, 2001). The observed increase Ig (s) class level in diabetics was in agreement with the results obtained by other research workers (Ritzmann and Daniels, 1975; Ardawi *et al*., 1994).

5.3. Autoantibodies (AAbs) in chronic diseases:

Autoimmune response (AIR) was the induction of an IR to self-Ags (Volpé, 1993; Bach, 1994). Chronic inflammation may be the prerequisite for the initiation and maintenance of the multi-step process leading to autoimmunity" (Di-Rosa and Barnaba, 1998; Hogenova *et al*., 1998). Higher prevalence of AAbs was observed among patients with chronic diseases [19.49% (77:395)], when compared to healthy controls [3.77% (4:106)] (tables- 11, 12, 13, 14, & 15). Such finding could be due to chronic inflammation; and tissue damage might be associated with disease chronicity (Dotsenko, 1993; Lindsay and Hoofnagle, 2000). Continuous exposure of cellular and tissue constituents to the immune component cells could mount an IR to them (Dotsenko, 1993; Willeox, 1993; Hogenova *et al*., 1998).

Chronic inflammation might favour priming of auto-reactive T-cells that have escaped thymic negative selection and are able to mount a cross-reactive response to self-mimicking properties (Di-Rosa and Barnaba, 1998). Moreover, chronic inflammation and persisting microbial infection can synergistically support autoimmunity through other relevant mechanisms (Di-Rosa and Barnaba, 1998); like:

1. Unveiling of cryptic self-epitopes.
2. Determinant spreading.
3. Activation of dendritic cells.
4. An ever priming of new auto-reactive T-cells.
5. Efficient generation and re-estimation of memory cells.

Viral infections may be associated with autoimmunity (Glynn, 1982). Chronic infections by HBV and HCV can be associated with extrahepatic manifestations (Hartman, 1997; Lohse *et al*., 1998; Goh *et al*., 1999). It was found that 18.27% (19:104) of patients with CVH-B or C have at least one type of the tested AAbs in their sera (table-15). This percentage was

lower than that observed by other researchers who found that 62-70% of patients with CVH-B&C have AAbs in their sera (Pawlotsky *et al*., 1994; Cacoub *et al*., 2000). This difference could be attributed in part to interferon therapy, as none of the patients under study received interferon or even had history of treatment with interferon (Pawlotsky *et al*., 1994; Czaja, 1997; Jablkowiski *et al*., 1997; Cacoub *et al*., 2000). The use of interferon for the treatment of CVH has become wide spread (Jablkowiski *et al*., 1997; Dumoulin *et al*., 1999). Autoimmunity seemed to be the most important side effect of interferon therapy (Pawlotsky *et al*., 1994; Fritzsch *et al*., 1997; Jablkowiski *et al*., 1997; Dumoulin *et al*., 1999; Cacoub *et al*., 2000). In addition to interferon therapy, this difference could be attributed to the difference in the nature of AAbs studied. However, concurrent presence of different AAbs might suggest the presence of more than one disease entity, or imply that microbial infection was an important etiologic basis for autoimmune expression (Czaja, 1994; Ferri and Zignego, 2000). Factors influencing the production of AAbs and the development of concurrent immune aberrations in CVH are unknown, but viral Ag(s) versus host response to that Ag(s) are undoubtedly important (Czaja, 1994; Ferri and Zinego, 2000).

Upon comparison between the AAbs prevalence among CVH-C and CVH-B patients, no significant difference was noted (tables- 11 & 15). This was in agreement with what have been reported by Czaja (1997) and Lohse *et al*., (1998), who mentioned that patients with CVH-B&C could have similar immune features. Meanwhile, CVH-C patients are more commonly undergoing autoantigen driven processes.

Whether a *Brucella* infection induces an AIR was not well documented. However, the present study shows somewhat significant number of chronic brucellosis patients express the presence of AAbs in their sera (table-11&14). This could be due to the similarity between an epitope(s) of an autoantigen and an epitope(s) in the *Brucella* organism or

that chronic immunologic activation can affect the tolerance to self components (Fournie *et al*., 1974; Hogenova *et al*., 1998).

The immune destruction of pancreatic beta cells probably involved both humoral and cell mediated mechanisms, the later being more important (Knip, 1997; Bello, 1998; Knip, 1998). Diabetics were associated with the presence of AAb(s) in their sera. Although both type-1 and type-2 diabetics express the presence of AAb(s), type-1 diabetics significantly associated with more AAb(s) prevalence than type-2 diabetics (Doniach *et al*., 1982; Wahbi, 1998; AL-Fakhar, 2000; Pietropaolo *et al*., 2000; Sherwin, 2000).

Variation in AAb(s) prevalence probably reflected differences among patients in their immune responsiveness to the same Ag(s) (Czaja, 1997).
Rheumatoid factor (Rf) was commonly present in chronic inflammatory reactions (Edmonds, 1985; Czaja, 1997). It was the most commonly detected autoimmune marker in all the tested patient's groups. This was in accordance with what had been reported by other research workers (Edmonds, 1985; Musset *et al*., 1993; Pawlotsky *et al*., 1995; Hartman, 1997; Nagao *et al*., 1997; Philips, 1997; Valentini *et al*., 1999; Eng *et al*., 2000; Hazleman, 2000; Seifert, 2000). Arthralgia and arthritis were parts of the common symptoms associated with the three tested diseases (Czaja, 1997; Cacoub *et al*., 2000; Hazleman, 2000; Seifert, 2000; AL-Yaquby, 2001; Taha, 2001). The deposition of immune complexes in the synovial fluid have been associated with the production of anti-globulin (Roitt *et al*., 1982). Among diseases other than rheumatoid arthritis associated with Rf seropositivity were viral hepatitis, chronic bacterial infection and D.M (Edmonds, 1985; Ognenovski and Mc Cune, 1998). The presence of Rf in non-rheumatic conditions was typically associated with hypergammaglobulinemia and chronic antigenic stimulation that resulted in polyclonal B-cell function. Under certain circumstances, it might represent anti-idiotypic Abs to Abs produced by the patients against Fc receptors of

bacteria, parasites, or viruses (EL-Shawarby *et al*., 1993; Czaja, 1997; Eng *et al*., 2000). Rf seropositivity was strongly associated with the presence of another AAb type(s), since more than half of subjects who were positive for Rf were also positive for another AAb type(s) [52.94%(27:51)].

The prevalence of AMAbs among test patient's groups was low (Shibata *et al*., 1993; Hoofnagle, 1997; Lohse *et al*., 1998). The AMAb showed high degree of specificity for primary biliary cirrhosis (~99%) (Doniach *et al*., 1982; Hyde, 2000; Roitt and Robson, 2000). However, only one patient of the 58 CVH-B patients expressed the presence of AMAb in his serum. This could reflect the possibility that HBV infection might be a trigger for the expression of primary biliary cirrhosis in this patient. Patients with CVH-C do not express the presence of AMAb in their sera (Lohse *et al*., 1998). This was in agreement with the results observed by Shibata and associates (1993) who revealed that the prevalence of HCV infection among patients with primary biliary cirrhosis was low. The presence of AMAb in chronic brucellosis patients [3.13% (1:32)] might be coincidental or that microbial chronic infection could induce the formation of these AAb (Hogenova *et al*., 1998).

Thyroid Abs (TgAb and/ or TPoAb) were observed in different rates in the three studied patient's groups. The high prevalence of thyroid Abs in diabetics especially type-1 diabetics came in accordance with the results of Kilburg *et al*., (1999); Lindberg *et al*., (1999); and Rennert and Francis, (1999). The high prevalence of thyroid Abs in type-1 diabetics put an emphasis on the importance of early screening for thyroid disease in type-1 diabetics (Lindberg *et al*., 1999; Lindberg *et al*., 2001). Among patients with CVH-B&C, the presence of thyroid Abs was confounded to CVH-C patients [4.35% (2:46)]. A finding that came in contrast with what has been reported by Deutch and associates (1997), who revealed that the overall prevalence of thyroid Abs among untreated patients with CVH-B, C, and D

was 14%. This difference could be attributed to the HDV superinfection state that might worsen disease prognosis (Levine *et al*., 1994). Thyroid Abs in series-I brucellosis patients was not observed. A finding that came in accordance with the observation of Madkour (2001 b) who mentioned that TPOAb and TgAb were not detected in patients suffering from thyroiditis due to *Brucella* infection (Vermiglio *et al*., 1995).

Anti-cardiolipin Ab (ACAb) was one of the naturally occurring AAbs, and was noted among patients with connective tissue disorders, infections, and drug induced disorders (O'Connell, 1980; Hansen, 1998; Eng *et al*., 2000; Farhat, 2000). The factors that induced the formation of ACAbs were rather unclear (Hansen, 1998). Infection might play a role by demonstration that immunization with bacteria or phospholipid protein could induce anti-phospholipid Abs formation (Hansen, 1998; Farhat, 2000).

Higher prevalence of ACAb was observed among diabetics, brucellosis, and CVH patients when compared to healthy controls (table-10). This might indicate that infections might trigger the production of these Abs (Hansen, 1998; Eng *et al*., 2000); or imply that these AAbs might develop as a consequence of an occult autoantigen release via tissue damage (Tochino, 1987; Czaja, 1994); or that some of these patients were suffering from concomitant *Treponema pallidium* infection, since syphilitic patients might have ACAb (O'Connell, 1980; Glynn, 1982; Hansen, 1998; Farhat, 2000). Ahmed and associates, (1999) revealed that the presence of ACAb in serum samples of diabetics might be associated with cardiovascular complications. Furthermore, Farhat (2000) showed that anti-phospholipid Abs may occur in association with autoimmune diseases.

The prevalence of ANA was higher among patients with chronic diseases when compared to healthy controls. The observed characteristic ANA prevalence among patients with CVH-B&C (4.18%) and especially those with CVH-C (8.7%) was in accordance with other research workers (EL-Shawarby *et al*., 1993; Czaja, 1997; Hartman, 1997; Nagao *et al*.,

1997; Lohse *et al*., 1998; Eng *et al*., 2000). Viral proliferation might provide an appropriate antigenic material from viral or host cell origin, or it might favor other infections that lead to liberation of nuclear Ags (Lambert and Dixon, 1970).

Fournie *et al*., (1974) observed the release of DNA in circulating blood and induction of anti-DNA Abs after the injection of bacterial lipopolysaccharide, that might explain the presence of ANA among brucellosis patients.

ANA among type-1 diabetics (9.4%) was higher than that observed in type-2 patients (2%). This was in accordance with the observation that type-1 D.M was an autoimmune disorder, however, AAbs in type-2 diabetics were also observed at a lower rate (Fallucca *et al*., 1997; Wrobewski *et al.*, 1998; Kilburg *et al*., 1999; AL-Fakhar, 2000). Autoimmune mediated beta cell destruction might lead to the liberation of nuclear Ags (AL-Fakhar, 2000).

5.4. Anti-streptolysin-O immune reactivity:

Streptolysin-O is among the invasive toxigenic factors produced by *Streptococcus pyogenes* during an infection process (O'Connell, 1980; Brooks *et al*., 1998). Traditionally, ASO is usable as an indicator for post-streptococcal rheumatic conditions (Ruoff, 1998). However, AL-Talib and associates (1989) have documented a raise in ASOT in tuberculus patients (AL-Talib *et al*., 1986). Thus, this may be attributed to share epitope of this toxin with an epitope of *Mycobacterium* or their products. Based on the same assumption, anti-streptolysin-O specific epitope(s) may share characters with *Brucella*, HCV, HBV, and/ or abnormal metabolic protein molecule (D.M). Such shared epitope can explain in part the raise up of ASOT in such conditions. Other possibility could be that food borne phytopeptide might act as shared epitope with that of streptolysin-O leading to such raised ASOT.

5.5. *Salmonella typhi*-O immune reactivity:

The immune reactivity to *S.typhi*-O among patients with D.M; CVH-B&C; and brucellosis could reflect an underlying *S.typhi* affection (table-17). However, an enhance response to gut associated Ags was demonstrated among patients with CVH (Bjorneboc *et al*., 1972; Trigger *et al*., 1972; Wright, 1982). Kupffer cells may fail to sequester Ags absorbed from the gut. This could be due to functional impairment which might occur as a result of hepatic damage or saturation of Kupffer cells by other Ags (Protell, 1971; Triger *et al*., 1972; Wright, 1982). Chronic liver exposure to affection in diabetics might be associated with the raise up of *S.typhi* Abs.

The localization of *Brucella* organisms in the liver may lead to its final term to the formation of suppurative large granuloma (Salata, 2000).

5.6. HBV and HCV seromarkers in the study groups:

HBsAg prevalence among healthy controls (4.72%) was in agreement with the results obtained by Mohammed (1996) who revealed that HBsAg prevalence among general Iraqi population was 5%. In addition, Hassan (1997) found that 3% (75:2500) of healthy blood donors in Babylon governorate were positive for HBsAg. The persistence of HBsAg among CVH-B patients for at least one year confirmed their chronic course of infection (Lindsay and Hoofnagle, 2000). The absence of HBsAg and other HBV seromarkers from sera of CVH-C patients might exclude the HBV coinfection among such patients (Goudeau and Dubois, 1995). The prevalence of HBsAg among series-I chronic brucellosis patients [3.13% (1:32)] was comparable to that observed among healthy controls. There was no significant difference in HBsAg prevalence among diabetics when compared to healthy controls. This was in agreement with Abu AL-Hab (1992). However, the higher prevalence of HBsAg among IDDM patients (6.67%), although not significant compared to NIDDM patients, could reflect their increased risk of acquiring viral infection either by hospitalization through improper use of unsterilized needle for administration of insulin, that is mean HBV infection in these patients most probably associated with diabetes rather than related to it (Abu AL-Hab, 1992; Wahbi, 1998).

The high prevalence of anti-HBc IgM (68.96%) in CVH-B patients compared to that obtained in type-1 diabetics (1.89%) and healthy controls (0.96%) was in agreement with what had been reported by other research workers who found that anti-HBc IgM was higher in CVH-B patients compared to healthy carriers (Aballi, 1994; Zgair, 1999). The presence of these Abs could reflect an active viral replication and an increased expression of HBcAg on the infected hepatocytes which could result in an

increased recognition and destruction of the infected hepatocytes by the cytotoxic T-cells since HBcAg was considered as the main target for cytotoxic T-cells (Mondelli *et al*., 1982; Di-Stasi *et al*., 1994). All anti-HBsAg positive individuals had history of HBV vaccination (Melnick, 1993).

Anti-HCV was detected in all CVH-C patients and in 1.89% (1:53) of type-1 diabetics (table-17). It was not detected in serum samples of individuals from the other test groups. Anti-HCV prevalence among Iraqi healthy blood donors and general Iraqi population was found to be around 0.5% (Mohammed, 1996). Furthermore, Hassan (1997) showed that anti-HCV prevalence among healthy blood donors in Babylon was 0.48% (12:2500). Such difference could be attributed to the difference in the number of individuals tested, and the higher anti-HCV prevalence in type-1 diabetics could also be attributed to the increased risk of HCV infection among them.

5.7. Humoral Immunology of:

5.7.1. CVH-B&C:

Patients with CVH-B&C could express abnormal immunological manifestations (Wright, 1982; Lohse *et al*., 1998; Simmonds *et al*., 1998; Cacoub *et al*., 2000). There may be a specific response to viral Ags (Shashikumar and Paund, 1992; Zgair, 1999; Lindsay and Hoofnagle, 2000), a non-specific response to Ags unrelated to the pathogenesis of liver disease (Protell *et al*., 1971; Bjorneboc *et al*., 1972; Triger *et al*., 1972; Wright, 1982; Craig *et al*., 2002; Shnawa and AL-Ameedi, 2002); an acute phase response (Kallio, 1997; Zhou and Fischer, 1999; Clyne and Olshaker, 1999; Craig *et al*., 2002); and an overall increase in Igs level (Musset *et al*., 1993; Nagao *et al*., 1997; Eng *et al*., 2000). In addition to an AIR with Rf

and ANA as the most prevalent AAbs observed (Dotsenko, 1993; EL-Shawarby *et al*., 1993; Pawlotsky *et al*., 1995; Hartman, 1997; Di-Rosa and Barnaba, 1998; Lohse *et al*., 1998; Valentini *et al*., 1999; Shnawa and AL-Ameedi, 2002). These findings underscore the non-specificity of the immune manifestations in the chronic liver disease (Czaja, 1997).

5.7.2. Chronic brucellosis:

Brucellosis is a systemic infection which may involve any organ with the non-specific symptoms (Young, 1983; Salata, 2000). Chronic brucellosis is now recognized as analogues to chronic fatigue syndrome, i.e. patients who feel chronically ill, fatigued and have elevated anti-*Brucella* Abs (Cluff, 1991). Arthralgia and fever are the major symptoms in the chronic form of the disease (Taha, 2001). However, all the patients involved in the present study have multiple joint pain, fever, and raised anti-*Brucella* Abs (titer ranging from 1:160 up to 1:640).

Major immune manifestations among such patients were increased CRP (62.5%) (table-6) and Igs level (table-7), which might reflect an acute flare up of the chronic course of the disease (Gazapo *et al*., 1989; Clyne and Olshaker, 1999; Young, 2001; Craig *et al*., 2002).

The observed AIR in about 21.8% of series-I patients and 14.1% of series-II patients, with Rf, AC, and ANA as the most prevalent AAbs detected (tables- 11 & 14), could indicate that there might be an epitope in *Brucella* organism that might trigger the autoimmune machinery or that chronic infection could initiate an autoimmune process (Fournie *et al*., 1974; Di-Rosa and Barnaba, 1998; Hogenova *et al*., 1998). In addition, a characteristic percentage (53.1%) of patients showed raised ASOT (table-16). This could reflect coexistence streptococcal infection, or that there might be sharing antigenic similarity between streptolysin-O and certain *Brucella* Ag(s). The immune reactivity to *S.typhi*-O (2:32) observed in

series-II chronic brucellosis patients could reveal that those patient(s) were suffering from coexistence typhoid fever; however, false positive reaction could not be excluded among such patients (Taha, 2001).

5.7.3. D.M

Diabetes mellitus (D.M) is a chronic disorder characterized by impaired metabolism of glucose and other energy- yielding fuels, as well as late developments of vascular and neuropathic complications (Belfiore and Ianello, 2000; Sherwin, 2000). Diabetics, both type-1 and type-2, express abnormal immune manifestations. Diabetics might exhibit hypergammaglobuliemia (Ritzmann and Daniels, 1975). Increased IgM level was observed to correlate inversely with increased disease duration, whereas IgG and IgA levels were found to have a positive correlation to the increased disease duration. At time of diagnosis of type-1 D.M, IgM levels often increased (Ardawi *et al*., 1994; Craig *et al*., 2002). In established disease, IgM level could be one half that of normal controls. IgA (~83%) and IgG (~35%) were elevated in diabetics (Ardawi *et al*., 1994).

Elevated CRP indicated the presence of an acute phase response (Ritchie *et al*., 1999; Craig *et al*., 2002). Ford (1999) reported that CRP concentrations were lowest among those individuals without diabetes and highest among those with newly or previously diagnosed diabetics. A comparable finding was observed in the present study, as 52% of diabetics showed raised CRP level above normal compared to only 3.77% of healthy controls (table-6). In addition, a significantly higher percentage of type-1 diabetics showed raised CRP compared to type-2 diabetics. Recent observations indicated that type-2 diabetes might be a disease of the innate immune system (Pietropaolo *et al*., 2000). Increased CRP was associated with hyperglycemia (Moran and Romero, 1999). Furthermore, increased CRP was associated with the presence of AAbs (Pietropaolo *et al*., 2000). This finding could have a direct implication for understanding the

autoimmune/ inflammatory mechanisms involved in the pathogenesis of hyperglycemia (Ebeling *et al*., 1999; Moran and Romero, 1999; Pietropaolo *et al*., 2000).

The present study showed that both type-1 and type-2 diabetics expressed an AIR (tables- 11 and 13). Type-1 was associated with more AIR (47.17%) than type-2 diabetics were. Autoimmunity played a major role for the development of type-1 diabetes and some role for type-2 diabetes (Doniach *et al*., 1982; Wahbi, 1998; AL-Fakhar, 2000; Pietropaolo *et al*., 2000; Sherwin, 2000). Rf and thyroid AAbs were the most prevalent AAb types followed by ACAb and ANA.

The high prevalence of Rf could support the inflammatory process involved in the pathogenesis of type-1 and type-2 D.M, as the presence of Rf could reflect the chronic inflammatory reactions (Czaja, 1997). Diabetics, especially type-1, were found to have significantly more thyroid AAbs in their sera than healthy matched controls (Doniach *et al*., 1982; Hasso, 1995; Lindberg, 1999). Although the presence of AAbs in an individuals was not sufficient to cause AID, AAbs are more important markers for detecting individuals at risk for developing clinical disease (Lindberg, 1999). Nowadays, thyroid AAbs are accepted as an important tool for identifying patients at risk of developing hypothyroidism (Lindberg, 1999). The most common concomitant autoimmune endocrine disease occurring among patients with IDDM is autoimmune thyroiditis (Doniach *et al*., 1982; Hasso, 1995; Lindberg, 1999).

The increased prevalence of ACAb was associated with increased incidence of vascular complications among diabetics (Ahmed *et al*., 1999). The rise in ASOT in diabetics could reflect the infectious etiology of diabetes.

3. Materials and Methods:

3.1. Subjects:

The study extended from the first of June, 2001, till the first of August, 2002. It was carried out on 395 patients and 106 controls. Their characteristics were shown in table-1.

The control group consisted of 106 apparently healthy subjects; 45 were females and 61 were males. The age range was between 18 and 70 years, with the mean age of 43 years.

The patients were divided into three groups:

1. Patients with CVH-B&C:

One hundred and four patients with CVH-B&C were enrolled in this study. Their age range was between 15 and 63 years. These patients were serologically and clinically confirmed to be chronically infected (Lindsay & Hoofnagle, 2000). Disease duration ranges from one year to ten years.

2. Patients with chronic brucellosis:

This group consisted of 188 patients who were confirmed to have chronic brucellosis (Cluff, 1991; Salata, 2000; Taha, 2001). Their age ranged from 15 to 55 years. There were 111 (59.04%) females and 77 (40.96%) males. The patients were divided into two series:

Series I consisted of thirty-two patients who were tested for all parameters.

Series II consisted of 156 patients, tested for RF only.

3. Patients with D.M:

A total of 103 D.M patients were included in this study (Sherwin, 2000). Their age ranged from 17 to 75 years. There were 53 (51.46%)

type-1 D.M. (IDDM) patients and 50 (48.54%) type-2 D.M. (NIDDM) patients.

3.2. Blood Samples:

A total of 501 blood samples were collected from 395 patients and 106 healthy individuals as a control group. From each individual, a blood sample of 5-10 ml was drawn aseptically in a disposable syringe. The blood samples were left at room temperature till clot, and then they were centrifuged at 3000 rpm for 15 minutes. The sera were aspirated from the whole blood, then divided into 0.5 ml small test tubes, and stored at –20 ºC till testing time. Each tube was used once to avoid repeated thawing and freezing. All sera and reagents were allowed to stand at room temperature before being used for the tests.

3.3. Pleural fluid samples:

Pleural fluid was obtained from patients suffuring from an acute infection.

3.4. Laboratory Animals:

Nine mature male local rabbits, *Oryctolagus cuniculus* (Hime & Donoghue, 1979) were the study laboratory animals. Each rabbit was about 2 kg of weight. These rabbits were left for two weeks for adaptation, checking for affections and kept *ad libitum* (Schneider *et al.*, 1990). The animals were divided into three groups; each group consisted of three replicates.

3.5. Equipment & Tools:

Equipments used during this work were :

The instrument	Company	Country
Autoclave	Webeco GmbH	Germany
Automatic pipettes	Organon Teknika	Belgium
Centrifuge	Damon-IEC-division	USA
Disposable petri dishes	Sterilin	England
Disposable syringes	Meheco	China
Disposable tips	Netheler-Hinz	Germany
Disposable Plastic tubes	AFMA-Dispo	Germany
Distiller	H-Jurgens & Co.	Germany
Deep Freezer of –18 to –20ºC	Ishtar	Iraq
Glass centrifuge tubes	Sterilin	England
Heating block	Organon Teknika	Belgium
Incubator	Memmert	Germany
Light microscope (complex)	Olympus	Japan
Microelisa aspiration/ washing System	Organon Teknika	Belgium
Microtitration Plate	Cook engineering Co-Alexandria	Virginia
Magnetic stirrer	Cole parmer	Chicago
Multi-channels pipette	Organon Teknika	Belgium

The instrument	Company	Country
Opacimeter	WHO Internationa Reference Preparation of Opacity/International laboratories for biologica standards & controls.	England
Optical Reader	Medic	Italy
Oven	Memmert	Germany
Pasteur pipette	Biomerieux	France
Petri – dishes	Sterilin	U.K.
pH meter	Philips	Holland
Reader micro ELISA system	Organon Teknika	Belgium
Refrigerator	Ishtar	Iraq
Sensitive balance	Sartorius	U.K.
Spectronic –20		U.K.
Sterile plastic test tubes	AFMA-Dispo.	Germany
Timer	Hener-Tractor, 7 Jewels	Swiss
Mixer	Griffin & George Ltd.	U.K.
Water bath	Memmert	Germany
Filter paper	Whatman , 0.22 Mm	Germany
Microfiltration unit	Cottingen / W	Germany
Glass slides and cover slips	Meheco	Germany

3.6. Solutions:

3.6.1. Normal saline:

The solution was prepared by dissolving 0.85 gm sodium chloride (Nacl; BDH company; U.K; M.W=58.44) in 20 ml distilled water; the volume was then completed to 100 ml. The final concentration was 0.85 %. The solution was then sterilized by autoclaving (121ºC/ 15 Bar/ 15 minutes). It was used in preparing formaline solution and for titration purposes.

3.6.2 Phenol saline solution:

This solution was prepared by dissolving 8.5 gm of Nacl and 5 gm phenol in 1000 ml distilled water. It was used for serum dilution in tube agglutination test.

3.6.3. Formaline solution:

The formaline solution was prepared by mixing 3.75 ml formaldehyde (40% concentration; H-CHO; BDH company; M.W=30.3) with 20 ml normal saline. The volume was then completed to 300 ml with normal saline (0.85%). The final formaldehyde concentration would be 0.5%. This solution was used to prepare heat-killed *E.coli* Ags (Lehmann *et al.*, 1968).

3.6.4. Tannic acid solution:

The solution was prepared by dissolving 0.5 gm tannic acid powder ($C_{76}H_{52}O_{46}$; BDH company; M.W=1701.22) in 20 ml distilled water. The volume was then completed to 100 ml with distilled water to obtain the final tannic acid concentration of 0.5%. This solution was used to tan RBCs i.e. to mope the Ags found on sheep erythrocytes surfaces (Garvey *et al.*, 1977).

3.6.5. Alsever's solution:

Alsever's solution is an isotonic, anticoagulant blood preserving solution that permits the storage of whole blood at refrigeration temperature for about 10 weeks (Talib, 1996). This solution was prepared by dissolving 24.6 gm glucose (BDH company); 9.6 gm tri-sodium citrate (BDH company) and 5.04 gm sodium chloride (BDH company) in 1200 ml distilled water; the pH was then adjusted to 6.1 with 15% citric acid. The solution then filtered through 0.22µm pore's diameter membrane filter using microfiltration unit.

3.6.6. Biuret solution:

This solution was used in Biuret method for estimation of protein concentration (Ross, 1985). It was prepared by dissolving 3 gm copper sulfate ($CuSO_4$. $5H_2O$; BDH company; M.W=166); 9 gm sodium-potassium tartarate ($NakC_4H_4O_6.2H_2O$; BDH company; M.W=166) and 5 gm potassium iodide (KI; BDH company) in 500 ml distilled water. To this solution, 100 ml sodium hydroxide (BDH company; 0.6 molar) was added. The volume was then completed to one liter with distilled water.

3.6.7. Standard concentrations of bovine albumin:

Serial double dilutions of standard concentrations of bovine albumin was used to construct the standard curve for protein concentration determination (Ross, 1985). Sixty gm of dry bovine albumin (BDH company; M.W=65400) were dissolved in a small amount of sodium hydroxide solution (0.6 molar). The volume was then completed to one liter with the same solution. The final protein concentration was equal to 60 gm/l. From this solution, two fold dilutions were prepared by using 0.6 M sodium hydroxide solution as a diluent to obtain the following titers: total, 2, 4, 8,

16, 32, 64 and 128 representing the following concentrations: 60, 30, 15, 7.50000, 3.75000, 1.87500, 0.93750 and 0.46875 gm/l, respectively.

3.7. Media:

3.7.1. Brain-heart infusion (Oxoid Company/ USA):

This medium was used for the revival of *E.coli* and *Streptococcus pneumoniae*. It was prepared according to the manufacturer's instructions.

3.7.2. Nutrient agar (Oxoid Company / USA):

This media was prepared according to the manufacturer's instructions. It was used for the cultivation of *E.coli* and *Streptococcus pneumoniae* .

3.8. Reagents:

3.8.1. Prepared reagents:

3.8.1.1. Biuret method for the determination of protein concentration in solutions:

The protein concentration in solutions was determined according to Biuret method, which has been described by Ross (1985). The procedure was carried out as followed:

Five ml of biuret solution were added to each of the control and the test tubes. To the control tube, 0.2 ml of distilled water was added, whereas to the test tubes 0.2 ml of the solution (to be tested) was added. Each tube was then mixed gently and left in a dark place at room temperature for 30 minutes. The optical density (O.D) of the test tube was then estimated, by using spectronic-20 at 540 nm after the standardization of the spectronic by using the control tube. Protein concentration in the test tube was determined by using the standard curve equation.

The O.D $_{540\ nm}$ of the previously prepared standard serial dilutions of a known concentration of bovine albumin (section 2.5.7) was obtained according to the method above, and the standard curve was constructed by plotting the O.D on the X-axis versus the corresponding concentration on the Y-axis. The best straight line was drowning as determined by the points that represent the O.D versus the corresponding concentration. Then the straight-line equation was determined:

$$\mathbf{Y = a + bx}$$

3.8.1.2. Standard curve for protein concentration determination:

Biuret method was used to estimate the O.D at 540 nm for the standard bovine albumin concentrations (Ross, 1985). The O.D values for the different concentrations of bovine albumin were shown in table-3. The simple regression analysis showed that standard curve equation was of simple first order equation, where y represented the protein concentration corresponding to the O.D value (x). The equation was as:

$$\mathbf{y= \ - 4.07203 + 147.07x}$$

There was a linear relationship between the O.D value (x) and the increasing concentration of the standard bovine albumin (y), hence the standard curve was plotted (figure-1).

Table-2: Statistical data corresponding to the standard bovine albumin concentrations.

A- Statistical data:

X	y	X^2	y^2	Xy
0.383	60.00000	0.146689	3600.0000	22.9800000
0.283	30.00000	0.080089	900.0000	8.4900000
0.170	15.00000	0.028900	225.0000	2.5500000
0.070	7.50000	0.004900	56.2500	0.5250000
0.050	3.75000	0.002500	14.0625	0.1875000
0.034	1.87500	0.001156	3.5156	0.0637500
0.029	0.93750	0.000841	0.8789	0.0271880
0.014	0.46875	0.000196	0.2197	0.0065625
Σx= 1.033	**Σy=119.525**	**Σx^2 = 0.265271**	**Σy^2=4799.9267**	**Σxy=34.83**
$\bar{x}$ **=0.129**	$\bar{y}$ **=14.900**			

x: optical density.

y: protein concentration.

B. The calculation of standard curve equation:

$$SSx = \sum x^2 - \frac{(\sum x)^2}{n} = 0.265271 - \frac{(1.033)^2}{8} = 0.1318849$$

$$SSy = \sum y^2 - \frac{(\sum y)^2}{n} = 4799.9267 - \frac{(119.525)}{8} = 3014.1485$$

$$SSxy = \sum xy - \frac{(\sum x)(\sum y)}{n} = 34.83 - \frac{(1.033)(119.525)}{8} = 19.396335$$

$$b = \frac{SSxy}{SSx} = \frac{19.396335}{0.1318849} = 147.07$$

$$a = \bar{y} - bx = 14.9 - (147.07)(0.129) = 14.9 - 18.97203 = -4.07203$$

$$\bar{y} = a + b\bar{x}$$

$\bar{y} = -4.07203 + 147.07x$ **Standard curve equation**

$$r = \frac{SSxy}{\sqrt{(SSx)(SSy)}} = \frac{10.396335}{\sqrt{(0.1318849)(3014.1485)}} = \frac{19.396335}{19.93792} = 0.973$$

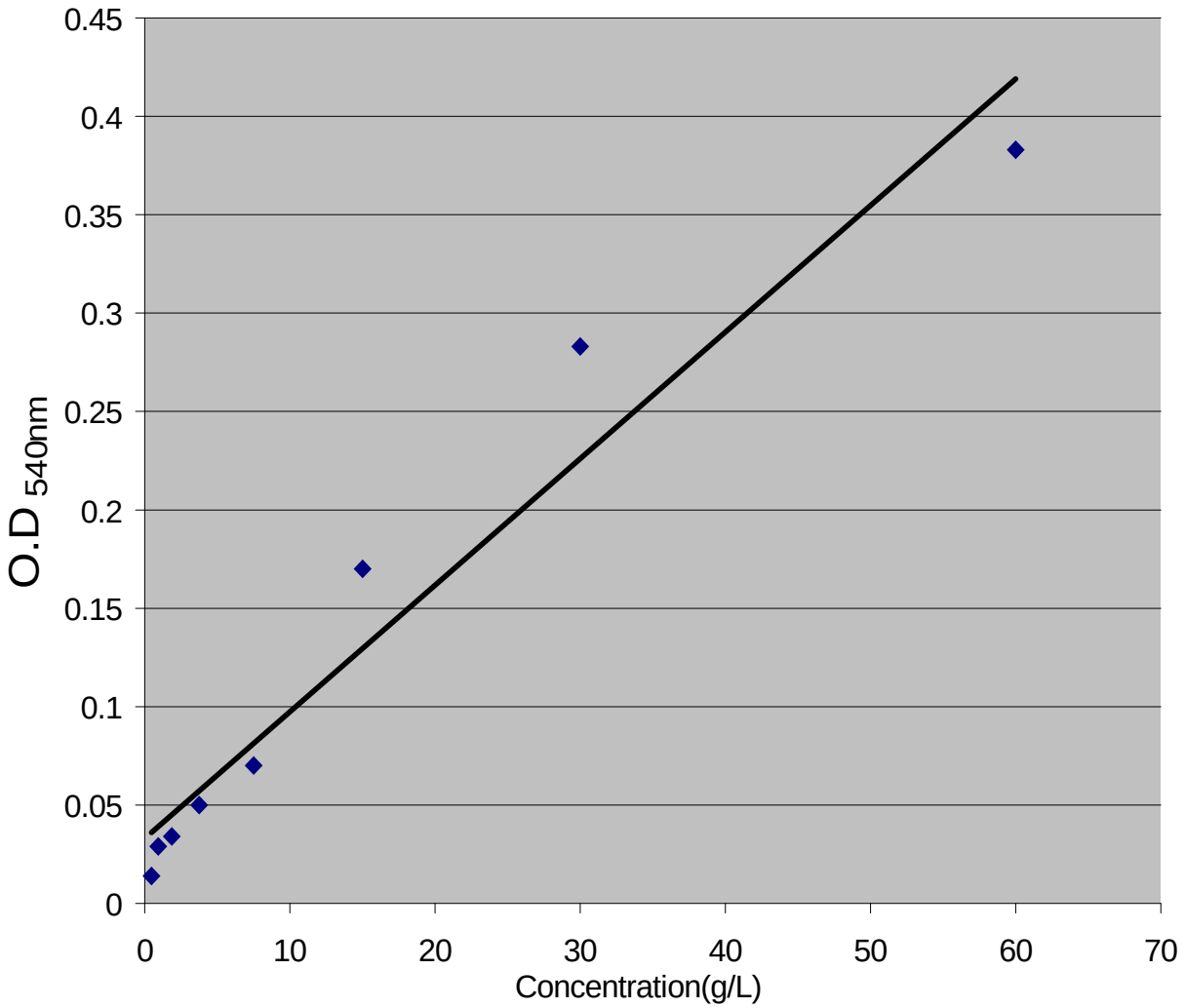

Figure-1: Linear relationship between the standard bovine albumin concentrations (X) and the O.D at 540 nm (Y).

3.8.1.3. Preparation of heat-killed *E.coli* Ag as microbial fraction of the adjuvant:

The surface Ags of *E.coli* was prepared according to the modified method of Smith (1975) and Svanborg-Eden *et al.*, (1985) as in the following steps:

1. *E.coli* culture isolated from urine sample of a patient suffering from urinary tract infection, was cultured on nutrient agar for 24 hours at 37ºC. Repeated culture was avoided.
2. Six ml of sterile saline solution was added to the growth surface and the growth was swiped by sterile loop.

3. The suspension was collected by sterile pipette, mixed by mixer for 3 minutes and centrifuged at 3500 rpm for 5 minutes.
4. The deposit was re-suspended in 5 ml sterile saline solution and centrifuged at 3500 rpm for 5 minutes. This step was repeated twice.
5. The deposit was re-suspended in 10 ml sterile saline solution.
6. From this suspension, 1ml was taken in the opacimeter tube and the volume was completed with formaline solution till the opacity became equal to that of the standard opacimeter tube (WHO standard) and thus the final concentration equal to 10 IU .
7. The suspension was then placed in water bath at 60ºC for 1.5 hour.
8. The solution (0.1 ml) was cultured on a nutrient agar plate and incubated at 37ºC for 72 hours. The culture yielded no growth.

3.8.1.4. Extraction of somatic c-polysaccharide of an R strain of *Streptococcus pneumoniae*:

Somatic c-polysaccharide of an R strain of *Streptococcus pneumoniae* was extracted according to the method of Tillet *et al.*, (1930). Five liters of brain heart infusion broth (Oxoid company) were inoculated with the bacteria and incubated at 37ºC for 48 hours. Bacterial cells were then collected by centrifugation at 5000 rpm for 10 minutes. The bacteria were re-suspended in 50 ml normal saline and were repeatedly frozen and thawed to break up the bacterial cell wall. To this solution of bacteria, 0.5 ml acetic acid (1 N, BDH company/UK) was added, and the mixture was then heated for 10 minutes in a boiling water bath. The preparation tube was cooled and the coagulated protein was removed by centrifugation at 2500 rpm for 3 minutes. Acidification and boiling were repeated to ensure the removal of all acid and heat precipitable material. The final water-clear supernatant fluid, neutralized with normal NaOH, contained c-polysaccharide of *Streptococcus pneumoniae.*

3.8.1.5. Precipitation of the CRP:

The crystallization of the CRP was carried out according to the modified method of Mc Carty (1947). Pleural fluid was used as the source material for CRP, rather than blood serum, as larger volumes of fluid could be obtained. In addition, pleural fluid's CRP free of lipid and by this character had facilitated the crystallization procedure (Mc Carty, 1947).

I. Initial Fractionation with ammonium sulfate:

One liter of pleural fluid was obtained from patients suffering from acute streptococcal pneumonia. The fluid was tested for CRP by latex agglutination test, and was found strongly positive. The fluid was then centrifuged at 2500 rpm for 5 minutes to remove the accumulated fibrin. To the clear supernatant fluid distilled water was added to obtain the final volume of one liter, and solid ammonium sulfate (BDH company) was added to half saturation (314 gm/l). The inactive precipitate was removed by centrifugation at 2500 rpm for 5 minutes . Solid ammonium sulfate (172 gm/l) was added to the filtrate to obtain 0.75 saturation. The solution was then allowed to stand at room temperature overnight. After centrifugation at 2500 rpm for 5 minutes, the precipitate was suspended in 200 ml distilled water. The obtained protein fraction was a clear amber solution and supposed to contain all the CRP of the original fluid. The solution was dialyzed (according to the method of Boyer, 1986) against tap water and then against 0.01% calcium chloride without the formation of any visible precipitate.

II. Precipitation with pneumococcal c-polysaccharide:

Twenty-five milliliters of pneumococcal c-polysaccharide solution was added to the dialyzed solution. The mixture was incubated at 37ºC for two hours and refrigerated overnight. The polysaccharide-protein precipitate was recovered by centrifugation at 2500 rpm for five minutes and washed

three times with normal saline containing 0.01% calcium chloride. The washed precipitate was suspended in 20 ml normal saline and brought into solution by the dropwise addition of saturated sodium citrate (about 1 ml). A small amount of insoluble material was removed by centrifugation at 2500 rpm for 3 minutes and discarded.

III. Precipitation:

The citrated solution (21 ml) was mixed with an equal volume of saturated sodium-sulfate solution (prepared and held at 37ºC for two hours). This step was included for its possible effect in causing further dissociation of the polysaccharide-protein complex by the action of high salt concentration. The half-saturated solution remained entirely clear. An additional 72 ml of saturated sodium citrate was added, bringing the final concentration to 0.75 saturation. A light amorphous precipitate was formed that was scanty and appeared as irregular needles.

IV. Reprecipitation:

The crystalline precipitate was recovered as completely as possible by centrifugation at 5000 rpm for 10 minutes and re-dissolved in 10 ml distilled water. Thirty ml of saturated sodium sulfate was added with the immediate formation of an amorphous precipitate which gradually expressed a crystalline character after incubation for several days at 37ºC. On this occasion, most of the formed crystals were of well formed, flat and rhomboid plates. Along with this, rarely seen few sparse of tiny needles were occasionally noted. The crystalline precipitate was recovered by centrifugation at 5000 rpm for 10 minutes and redissolved in 20 ml distilled water. Further recrystallization of the material by the same procedure was not carried out.

3.8.1.6. Preparation of tanned erythrocytes:

Sheep blood (10 ml), which was obtained from the animal under sterile conditions, was mixed with an equal volume of Alsever's solution. The resulting mixture was kept at 4ºC. From this mixture, 3 ml was taken in a sterile centrifuge tube and centrifuged at 2500 rpm for five minutes. Using sterile Pasteur's pipette, the supernatant was discarded and the deposit was resuspended in ten ml normal saline and centrifuged at 2500 rpm for five minutes. The supernatant was discarded again and the deposit was resuspended in ten ml normal saline and mixed thoroughly.

From this mixture, three ml was taken in a sterile glass tube into which three ml of 0.5% tannic acid solution was added, mixed, and placed in the water bath at 37ºC for 10 minutes. The centrifugation was carried out at 2500 rpm for five minutes. The supernatant was discarded and to the precipitate three ml of normal saline was added and gently mixed.

3.8.2.Ready made reagents:

3.8.2.1. Single radial immunodiffusion (SRID) test kits:

Kallestad endoplate SRID test kits were used for the quantitative determination of human immunoglobulin IgA, IgG, and IgM in human serum (Sanofi Diagnostic Pasteur, USA).

3.8.2.2. ELISA test kits:

1.Hepanostika HBsAg microelisa system, for detection the of HBsAg in human serum or plasma. Organon teknika, Belgium.

2.Hepanostika anti-HBc IgM microelisa system for the detection of IgM-class Abs to hepatitis-B core antigen (anti- HBc IgM) in human serum or plasma. Organon teknika, Belgium.

3.Hepanostika anti-HBs microelisa system for the detection of Abs to hepatitis B surface antigen (anti-HBs) in human serum or plasma. Organon teknika, Belgium.

4.Bioelisa HCV (third generation ELISA tests) for the detection of Abs to HCV (anti-HCV) in human serum or plasma. Biokit, Spain.

5.Anti-mitochondria, microplate EIA, for the qualitative detection of AMAbs in human serum or plasma by indirect enzyme immunoassay. Sanofi Diagnostic Pasteur, USA.

6.Anti-thyroglobulin microplate EIA for the qualitative detection of ATgAbs in human serum or plasma by indirect enzyme immunoassay, Sanofi Diagnostic Pasteur, USA.

7.Anti-thyroid peroxidase microplate EIA for the qualitative detection of ATPoAbs in human serum or plasma by indirect enzyme immunoassay, Sanofi Diagnostic Pasteur, USA.

8.Anti-cardiolipin microplate EIA for the detection of AC AAbs in human serum or plasma by indirect enzyme immunoassay, Sanofi Diagnostic Pasteur, USA.

9-Anti-nuclear antibodies microplate EIA for the determination of ANA in human serum or plasma by indirect enzyme immunoassay, Sanofi Diagnostic Pasteur, USA.

3.8.2.3 . Latex agglutination test kits:

1. Rheumajet Rf for the determination of RF in serum by agglutination of latex particles on slide. Biokit, Spain.

2. Rheumajet CRP for the determination of CRP in human serum by agglutination of latex particles on slide. Biokit, Spain.

3. Anti-streptolysin-O kit for the determination of antistreptolysin-O antibody titer (ASOT) by slide or tube agglutination method. Omega Diagnostic limited/ U.K.

3.8.2.4. Tube agglutination test kits:

A. *Salmonella typhi*-O Ags: It was a suspension of formaline killed *S.typhi*-O, obtained from the Institute of Sera and Vaccines, Baghdad, to detect Abs to *S.typhi*-O in serum samples.

B. *Brucella* Ag for tube agglutination test: It was a suspension of formalin-killed *B.abortus* strain 99 obtained from the Institute of Sera and Vaccines, Baghdad for the detection of Abs to *B. abortus* in serum samples.

3.8.2.5. Alanine aminotransferase (ALT) level estimation kit:

Alanine aminotransferase (ALT) level estimation kit (Randox, U.K.) was used to estimate ALT level in serum samples by using a colorimetric method.

2.8. Immunization Protocol for the Preparation of Rabbit's Anti-CRP Antisera:

The laboratory animals were divided into three groups, each with three replicates. Multi-site injection protocol was used as described in table-2 (AL-Shahery and Shnawa, 1989; Shnawa and Thwaini, 2002). Cardiac puncture was performed to obtain rabbit's blood, collected in 10ml sterile tubes, left at room temperature till clot, and centrifuged at 3000 rpm for 5 minutes. The sera were aspirated from whole blood by sterile Pasteur's

pipettes and divided into sterile tubes of 0.5 ml each and stored at –20ºC till examination.

Table –3: Immunization Programme.

Time	Group-I	Group-II	Group-III (control)	Site of injection
1st & 2nd weeks	Adaptation *ad libitum*			
3rd week	1 ml standard	1 ml prepared	2 ml normal	1 ml intramuscular 0.5 ml pelvic

	CRP + 1 ml complete adjuvant*	CRP + 1 ml complete adjuvant*	saline	(subcutaneous) 0.5 ml subclavial (subcutaneous)
4th week	1 ml standard CRP + 1 ml incomplete adjuvant**	1 ml prepared CRP + 1 ml incomplete adjuvant**	2 ml normal saline	1 ml pelvic (subcutaneous) 0.5 ml cervical (subcutaneous) 0.5 ml subclavial (subcutaneous)
5th&6 th weeks	Leave			
7th weeks	Cardiac puncture			

***** Complete adjuvant analogous to Freund complete adjuvant and composed of one volume (5 ml) heat-Killed *E. coli* Ag mixed with one volume (5 ml) sunflower oil.

****** Incomplete adjuvant (sunflower oil only).

3.10. Laboratory Investigations:

3.10.1. SRID test:

To estimate the Igs class (IgA, IgG, and IgM) level in patients and controls sera, SRID test kits (Sanofi) were used according to Mancini *et al.*, (1965). There was a linear relationship existing between the Ag concentration and the corresponding squared immune precipitin ring diameter that formed in an Ab gel system.

The test procedure was carried out as follows:

Five µl serum from each individual were dispensed into wells of the plate containing agarose gel mixed with a monospecific antiserum. The plate then covered and incubated at room temperature on a level surface for 48 hours for IgA and IgG estimation, and for 72 hours for IgM estimation. The sample diffuse radically through the gel and the Ag form a precipitin ring with the monospecific antiserum. The immuno-precipitation ring diameter was measured by the optical reader and the relevant concentration corresponding to the precipitate's ring diameter was calculated from the conversion table provided with the kit.

3.10.2. ELISA test:

The procedures used in the ELISA tests were carried out according to the manufacturer's instruction.

ELISA method is used to detect the presence of HBsAg and specific Abs to HBcAg, HBsAg, HCV, mitochondria, thyroglobulin, thyroid peroxidase, cardiolipin, and nuclear antigens in the sera samples of tested individuals.

The principle of the test could be summarized as follows:

ELISA was an immunoenzymatic method in which the wells of a microtiter plate were coated with specific Ags or with specific Abs to detect the presence of the specific Abs (anti-HBcAg, anti-HBs, anti- HCV, anti-mitochondria, anti-thyroglobulin, anti-thyroid peroxidase, anti-cardiolipin, and anti-nuclear Abs), or the specific Ags (HBsAg), respectively.

Serum samples were added to these wells. If the specific Abs or the specific Ags were present in the serum sample, they would form stable Ag-Ab complexes on the well. After washing to remove the unbound material, anti-human IgG or anti-HBs labeled with an enzyme was added and, if the

Ag-Ab complex were present, the conjugate would bind to the complex. After a second wash , an enzyme substrate solution was added. This solution would develop a certain colour (according to the kit used) if the sample was positive. Wells containing negative samples remained colourless.

3.10.3. Latex agglutination test:

Latex agglutination test was used to detect the presence of Ags (or Abs) in serum samples, using latex particles coated with specific Abs (or Ags). The latex particles were suspension of polystyrene particles of a uniform size that allowed visual observation of the Ag-Ab reaction, because the latex suspension would change its uniform appearance and an agglutination became evident. However, the uniform appearance indicated a negative result.

3.10.3.1. Detection of rheumatoid factor (Rf, anti-IgG Ab):

The presence of Rf in the sera of the tested individuals was evaluated by using rheumajet Rf kit (Biokit, Spain). Latex reagent was a suspension of polystyrene latex particles coated with human gamma globulin. The latex reagent (50 µ1) was mixed with an equal volume of serum. If the serum contained approximately more than 10 IU/ml of the Rf, a clear agglutination would appear within two minutes. Results were expressed in International Unit per ml (IU/ml) based on the International Reference Preparation of Rheumatoid Arthritis Serum (WHO).

3.10.3.2. Detection of CRP:

The presence of CRP in the sera of the tested individuals was evaluated by using rheumajet CRP (Biokit, Spain), which is a rapid test for

the qualitative and semi-quantitative determination of CRP in serum by agglutination of latex particles on slide.

The latex reagent (a suspension of polystyrene latex particles with uniform size coated with the IgG fraction of anti-human CRP specific serum) was mixed with an equal volume (50 µl) of serum on a slide. If the serum contained approximately more than 6 mg/L of CRP, a clear agglutination would appear within two minutes. Results were expressed in mg/L based on the WHO International Standard for human CRP.

For the semi-quantitative technique, serial double dilutions of the serum were prepared and the procedure was carried out as above. The approximate concentration would be equal to the reciprocal of the highest dilution that would present a clearly visible agglutination multiplied by 6.

3.10.3.3. Determination of ASOT:

For the determination of ASOT in the sera of tested individuals, ASOT kit (Omega Diagnostic Limited, U.K) was used. The latex reagent (latex particles coated with streptolysin-O) was mixed with an equal volume of serum. If the reaction took place due to the presence of anti-streptolysin-O in the serum sample, a clear agglutination became evident. Positive results were further tested by the same procedure after preparing serial double dilutions to determine the highest dilution that still present a clearly visible agglutination.

3.10.4. Tube agglutination test:

Formaline killed *B.abortus* and *S.typhi*-O suspension, prepared by the Institute of Sera and Vaccines, Baghdad, was used to detect the presence of Abs to *B.abortus* and *S.typhi*-O in test serum, respectively. The test was performed according to the technique described by Alton and Jones in1967 (Alton *et al*., 1988) as follows:

1.Eight small plastic tubes were used for each test sample.

2.In the first tube 800 µl of phenol saline solution and 500µl in each preceding tube (2 to 8) was drawn.

3.Then 200 µl of the test serum was added to the first test tube and mixed well.

4.From the mixture of the first test tube 500 µl was transferred to the second one, mixed well, and so on preceding until reaching the last test tube.

5.From the last tube 500 µl was discarded.

6.Five hundreds µl of the Ag was added to each tube, the dilutions thus obtained were 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640 and 1:1280, accordingly.

7.The plastic tubes were then covered with parafilm andincubated at 37ºC, for 24 hours. Afterwards, the results were read.

8.The appearance of clumps in the bottom of the tube with irregular edges and clear upper layer of the supernatant indicated positive results. If negative results were obtained, further 24 hours incubation was carried out to achieve a stable end point (Young, 1991).

3.10.5. Passive haemagglutination test (PHA):

According to the modified method of Gravey *et al.*, (1977), PHA was used to determine anti-prepared and anti-standard CRP level in rabbit's sera after the immunization programme. It was also used to determine CRP titer in 50 randomly selected patients and controls (ten from each study group).

3.10.5.1. Determination of anti-prepared CRP antibodies level in rabbit's sera:

Tanned sheep erythrocytes (3 ml) were mixed with one ml of the prepared CRP and left at room temperature for 10 minutes. After centrifugation at 2500 rpm for five minutes the supernatant was discarded and to the deposit three ml normal saline was added. Then centrifugation at 2500 rpm for five minutes was carried out. The supernatant was discarded and to the deposit four ml of normal saline was added and mixed. Microtitration plates were used to estimate anti-prepared CRP Ab level in rabbit's sera, according to the method of Garvey *et al.*, (1977).

The procedure was carried out as follows:

1. Fifty µl normal saline was dispensed in each well of the microtitration plate wells (12 wells).
2. Ten µl of rabbit's anti-prepared CRP antisera was added to 990 µl normal saline in a sterile test tube and mixed well.
3. Fifty µl of diluted rabbit's serum (1:100) was added to the first well and mixed. Then serial dilutions were prepared by transferring fifty µl from the first well to the second one, mixed well and from it fifty µl was transferred to the third well and so on. From the eleventh well fifty µl was discarded. Serial dilutions thus was obtained from the first well till the eleventh one: 1:200, 1:400, 1:800, 1:600, 1:3200, 1:6400, 1:12800, 1:25600, 1:51200, 1:102400, and 1:204800, accordingly.
4. To the twelfth well, fifty µl of tanned sheep erythrocytes covered with the prepared CRP was added (the last well represented the negative control well which was lacking the presence of rabbit's anti-prepared CRP Abs).
5. The microtitration plates was shaken gently for about two minutes and then incubated at 37^{o}C for 2 hours. Then the presence or absence of haemagglutination was recorded. PHA titer represented the last dilution that gave a positive result.

3.10.5.2. Determination of anti-standard CRP Abs level in rabbit's sera:

The procedure was carried out as described earlier in section (2.9.5.1) except that the tanned erythrocytes were coated with standard CRP instead of prepared CRP, and that the rabbit's anti-standard CRP antisera was used instead of rabbit's anti-prepared CRP antisera.

2.9.5.3. Determination of CRP titer by PHA:

PHA was used to estimate CRP titer in 50 selected individuals (10 from each study group) by using:

A. Tanned coated erythrocytes with rabbit's anti-prepared CRP antisera:

The procedure was carried out as described earlier in section 2.9.5.1 except that the tanned coated sheep erythrocytes were coated with 1 ml rabbit's anti-prepared CRP after decomplementization at 56ºC for 30 minutes, instead of prepared CRP, and that the undiluted prepared CRP was used instead of diluted rabbit's anti-prepared CRP antisera. Serial dilutions was thus obtained from the first well till the eleventh one; 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, and 1:2048 accordingly.

The last well represents the negative control well, which was lacking the presence of standard CRP.

B. Tanned coated sheep erythrocytes with rabbit's anti-standard CRP antisera:

The procedure was carried out as described earlier in section 2.9.5.1 except that the tanned sheep erythrocytes were coated with 1 ml rabbit's anti-standard CRP (after decomplementization at 56ºC for 30 minutes) instead of prepared CRP, and that the undiluted standard CRP was used instead of diluted rabbit's anti-prepared CRP antisera.

Two fold dilutions were thus obtained from the first well till the eleventh one; 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, and 1:2048 accordingly. The last well represented the negative control well, which was lacking the presence of standard CRP.

3.10.6. Estimation of alanin aminotransferase (ALT) level:

Alanine aminotransferase (ALT) level in serum samples of patients with CVH-B&C was estimated by the colorimetric method of Reitman and Frankle (1957).

2.11. Statistical analysis:

The Chi-squared test (χ^2) was used when the observations were countable (frequencies). The correlation coefficient (r) was used to determine the degree of the relationship between variables (Zar, 1999). P-values less than or equal to 0.05 were considered significant.

Table-1: The characteristics of the studied groups.

Group	Number	Age range (years)	Sex		Disease duration (years)	Investigation results	Diagnosis reference
			Male	Female			
CVH	104	15-63	62.5% 65:104	37.5% 39:104	1-10	•CVH-B: Positive for HBsAg for more than 6 months & ALT>20 U/L [58:104 (55.77%)] •CVH-C: Positive for anti-HCV & ALT level > 20 IU/L [46:104 (44.23%)]	Lindsay & Hoofnagle, 2000.
188	15-55	40.96% 77:188	59.04% 111:188	1-8	Symptoms of Pyrexia of unknown Origin. Recurrent fever. Arthralgia. Positi	Cluff, 1991; Salata, 2000; Taha, 2001.	D.M **103** **D.M**

					ve tube agglutination test for *B.abortus* up to 640.		
Controls	106	18-70	57.5% 61:106	42.5% 45:106		No history of viral hepatitis, diabetes and brucellosis infection.	

CVH: Chronic Viral hepatitis; **CVH-B:** Chronic viral hepatitis type-B; **CVH-C:** Chronic viral hepatitis type-C; **D.M:** diabetes mellitus; **IDDM:** insuline dependent diabetes mellitus; **NIDDM:** non-insuline dependent diabetes mellitus. ALT: alanine aminotransferase.

Table-6: CRP concentration in the studied patients groups using latex agglutination test.

CRP concentration (mg/L)	**CVH**			**Chronic brucellosis**	**D.M**			**Healthy controls**
	CVH-B	**CVH-C**	**Total**		**Type-1**	**Type-2**	**Total**	
	No:total (%)	**No:total (%)**	**No:total (%)**	**No:total (%)**	**No:total (%)**	**No:total (%)**	**No:total (%)**	**No:total (%)**
<6	44:58 (75.86%)	33:46 (71.74%)	77:104 (74.04%)	12:32 (37.5%)	20:53 (37.74%)	29:50 (58%)	49:103 (47.57%)	102:106 (96.23%)
6	3:58 (5.17%)	1:46 (2.17%)	4:104 (3.85%)	0:32 (0%)	1:53 (1.89%)	0:50 (0%)	1:103 (0.97%)	2:106 (1.89%)

12	1:58 (1.72%)	2:46 (4.35%)	3:104 (2.89%)	0:32 (0%)	3:53 (5.66%)	1:50 (2%)	4:103 (3.88%)	1:106 (0.94%)
24	1:58 (1.72%)	2:46 (4.35%)	3:104 (2.89%)	3:32 (9.38%)	5:53 (9.43%)	1:50 (2%)	6:103 (5.83%)	1:106 (0.94%)
48	3:58 (5.17%)	2:46 (4.35%)	5:104 (4.81%)	8:32 (25%)	8:53 (15.09%)	10:50 (20%)	18:103 (17.48%)	0:106 (0%)
96	4:58 (6.90%)	4:46 (8.70%)	8:104 (7.7%)	6:32 (18.75%)	5:53 (9.43%)	4:50 (8%)	9:103 (8.74%)	0:106 (0%)
192	2:58 (3.45%)	2:46 (4.35%)	4:104 (3.85%)	2:32 (6.25%)	7:53 (13.21%)	4:50 (8%)	11:103 (10.68%)	0:106 (0%)
384	0:58 (0%)	0:46 (0%)	0:104 (0%)	1:32 (3.125%)	4:53 (7.55%)	1:50 (2%)	5:103 (4.85%)	0:106 (0%)

Table-11: Autoantibodies (AAbs) prevalence in the studied groups.

Autoantibody Type	**CVH**			**Chronic brucellosis seriesI**	**D.M**			**Healthy controls**
	CVH-B	**CVH-C**	**Total**		**Type-1**	**Type-2**	**Total**	
	No:total (%)	**No:total (%)**	**No:total (%)**	**No:total (%)**	**No:total (%)**	**No:total (%)**	**No:total (%)**	**No:total (%)**
Rf	7:58 (12.07%)	7:46 (15.22%)	14:104 (13.46%)	4:32 (12.5%)	19:53 (35.85%)	11:50 (22%)	30:103 (29.13%)	3:106 (2.83%)

AMAb	1:58 (1.72%)	0:46 (0%)	1:104 (0.96%)	1:32 (3.13%)	0:53 (0%)	0:50 (0%)	0:103 (0%)	0:106 (0%)
ATPoAb	0:58 (0%)	1:46 (2.17%)	1:104 (0.96%)	0:32 (0%)	6:53 (11.32%)	2:50 (4%)	8:103 (7.77%)	1:106 (0.94%)
ATgAb	0:58 (0%)	1:46 (2.17%)	1:104 (0.96%)	0:32 (0%)	6:53 (11.32%)	2:50 (4%)	8:103 (7.77%)	1:106 (0.94%)
ACAb	1:58 (1.72%)	1:46 (2.17%)	2:104 (1.92%)	2:32 (6.25%)	5:53 (9.43%)	2:50 (4%)	7:103 (6.8%)	1:106 (0.94%)
ANA	1:58 (1.72%)	4:46 (8.70%)	5:104 (4.81%)	2:32 (6.25%)	5:53 (9.43%)	1:50 (2%)	6:103 (5.83%)	1:106 (0.94%)

CVH: Chronic Viral hepatitis; **CVH-B:** Chronic viral hepatitis type-B; **CVH-C:** Chronic viral hepatitis type-C; **D.M:** diabetes mellitus; **Rf:** Rheumatoid factor; **AMAb:** Anti- mitochondrial antibody; **ATPoAb:** Anti- thyroid peroxidase antibody; **ATgAb:** Anti- thyroglobulin antibody; **ACAb:** Anti-cardiolipin Ab; **ANA:** Antinuclear antibody.

Table-13: Autoantibodies patterns in diabetics.

Autoantibody Type	**Patterns**										**Total**
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**X**	
Rf	–	–	+	+	+	+	+	+	–	–	30:103 (29.1%)
AMAb	–	–	–	–	–	–	–	–	–	–	0:103 (0%)
ATPoAb	–	+	–	–	+	–	–	+	–	–	8:103 (7.8%)

ATgAb	–	–	–	+	–	–	–	+	–	+	8:103 (7.8%)
ACAb	+	–	+	-	–	–	–	–	+	–	7:103 (6.8%)
ANA	+	–	–	–	–	+	–	–	–	–	6:103 (5.83%)
Type-1	1:53 (1.89%)	2:53 (3.77%)	2:53 (3.77%)	3:53 (5.66%)	2:53 (3.77%)	4:53 (7.55%)	6:53 (11.32%)	2:53 (3.77%)	2:53 (3.77%)	1:53 (1.89%)	25:53 (47.1%)
Type-2	0:50 (0%)	1:50 (2%)	0:50 (0%)	2:50 (4%)	1:50 (2%)	1:50 (2%)	7:50 (14%)	0:50 (0%)	2:50 (4%)	0:50 (0%)	14:50 (28%)
Total	1:103 (0.97%)	3:103 (2.91%)	2:103 (1.94%)	5:103 (4.85%)	3:103 (2.91%)	5:103 (4.85%)	13:103 (12.62%)	2:103 (1.94%)	4:103 (3.88%)	1:103 (0.97%)	39:103 (37.8%)

Rf: Rheumatoid factor; AMAb: Anti- mitochondrial antibody; ATPoAb: Anti- thyroid peroxidase antibody; ATgAb: Anti- thyroglobulin antibody; ACAb: Anti-cardiolipin Ab; ANA: Antinuclear antibody.

Table-14: Autoantibodies patterns in series-1 chronic brucellosis patients.

Auto antibody	**Patterns**						**Total**
	I	**II**	**III**	**IV**	**V**	**VI**	
Rf	-	+	+	-	+	-	4:32 (12.5%)
AMAb	-	-	-	+	-	-	1:32 (3.13%)

ATPoAb	-	-	-	-	-	-	0:32 (0%)
ATgAb	-	-	-	-	-	-	0:32 (0%)
ACAb	+	-	-	-	+	-	2:32 (6.25%)
ANA	-	-	+	-	-	+	2:32 (6.25%)
Total	1:32 (3.13%)	2:32 (6.25%)	1:32 (3.13%)	1:32 (3.13%)	1:32 (3.13%)	1:32 (3.13%)	7:32 (21.88%)

RF:Rheumatoid factor; **AMAb:** Anti-mitochondrial antibody; **ATPoAb:** Anti-thyroid peroxidase antibody; **ATgAb:** Anti- thyroglobulin antibody; **ACAb:** Anti-cardiolipin antibody; **ANA:** Anti-nuclear antibody**.**

Table-15: Autoantibodies patterns among patients with CVH-B&C.

Autoantibody	**Patterns**							**Total**
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	
Rf	+	+	-	-	+	-	-	14.104 (13.46%)
AMAb	-	-	+	-	-	-	-	1:104 (0.96%)
ATPoAb	-	-	-	-	-	+	-	1:104 (0.94%)

ATgAb	+	-	-	-	-	-	-	1:104 (0.94%)
ACAb	-	-	-	+	-	-	-	2:104 (1.92%)
ANA	-	+	-	-	-	-	+	5:104 (4.81%)
CVH-B	0:58 (0%)	1:58 (1.72%)	1:58 (1.72%)	1:58 (1.72%)	6:58 (10.35%)	0:58 (0%)	0:58 (0%)	9:58 (15.52%)
CVH-C	1:46 (2.17%)	3:46 (6.52%)	0:46 (0%)	1:46 (2.17%)	3:46 (6.52%)	1:46 (2.17%)	1:46 (2.17%)	10:46 (21.74%)
Total	1:104 (0.96%)	4:104 (3.85%)	1:104 (0.96%)	2:104 (1.92%)	9:104 (8.65%)	1:104 (0.96%)	1:104 (0.96%)	19:104 (18.27%)

CVH: Chronic Viral hepatitis; **CVH-B:** Chronic viral hepatitis type-B; **CVH-C:** Chronic viral hepatitis type-C; **RF:** Rheumatoid factor; **AMAb:** Anti-mitochondrial antibody; **ATPoAb:** Anti-thyroid peroxidase antibody; **ATgAb:** Anti-thyroglobulin antibody**; ACAb:** Anti-cardiolipin antibody; **ANA:** Anti-nuclear antibody.

Table-16: Antistreptolysin-O titer (ASOT) among patient groups and healthy controls.

Study group		**ASOT > 1:200**				
		No.:total	**%**	**Range**	**Median**	**Mean**
D.M	**Type-1**	22:53	41.5	200-800	500	463.6
	Type-2	9:50	18.0	200-800	500	422.2

	Total	31:103	30.1	200-800	500	422.9
CVH	**CVH-B**	4:58	6.9	200-600	400	400
	CVH-C	2:46	4.4	200-600	400	400
	Total	6:104	5.8	200-600	400	400
Chronic brucellosis		17:32	53.1	400-800	600	541.2
Controls		5:106	4.7			200

D.M: diabetes mellitus; **CVH:** Chronic Viral hepatitis; **CVH-B:** Chronic viral hepatitis type-B; **CVH-C:** Chronic viral hepatitis type-C.

Table-17: *Salmonella typhi* immune reactivity among patient groups and healthy controls.

Patient group	**Anti-*S.typhi* Ab**		
	1:80	**≥1:160**	**Total**

		No.:total	%	No.:total	%	No.:total	%
D.M	**Type-1**	6:53	11.32	4:53	7.55	10:53	18.87
	Type-2	2:50	4.0	4:50	8.0	6:50	12.00
	Total	8:103	7.77	8:103	7.77	16:103	15.53
CVH		0:104	0.00	7:104	6.73	7:104	6.73
Brucellosis		1:32	3.125	1:32	3.125	2:32	6.25
Healthy controls		1:106	0.94	0:106	0.00	1:106	0.94

D.M: diabetes mellitus; **CVH:** Chronic Viral hepatitis.