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Comparative analysis of immunological markers of leptospirosis and other bacterial infections among normal, high risk and unclassified categories of human flow in hospitals

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Abstract

The detailed information about the patients with bacterial and other infections are required to ensure the appropriate choice of treatment. Although white blood cell counts and C-reactive protein are the major immunological markers, the biomarkers elevates in acute and chronic stages of infectious diseases. In most cases, these biomarkers are providing pivotal importance to determine the etiological agent and also to differentiate bacterial and viral infections. A total of 175 confirmed bacterial including leptospirosis and 45 viral infectious blood samples and 55 healthy control samples were included. Initially total WBC counts and CRP levels were determined, further neutrophils were derived by following standard procedure. The increasing levels of TNF-α were observed in both types of infection and variations were observed among IL-2, IL-4, IL-8, IL-10 and IL-12 in bacterial and viral infections even changes found in day of infections also. On comparing with infectious state, leptospirosis of both acute and chronic stages showed high elevation of CRP levels. Thus the levels of WBC counts, CRP and IL-2, IL-4, IL-8, IL-10 and IL-12 are potentially able to differentiate between bacterial and viral infections.

Keywords: Infections, Bacterial, Viral, WBC, CRP, cytokines, biomarkers.

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INTRODUCTION

In general, the pattern recognition molecules of white blood cells (WBC) are characterized as recognition at the time of the invasion of the pathogens; where these cells are responding to chemotactic signals present at the site of infection and inflammation [1]. Neutrophils are the host immune defence cells that are first to migrate into the skin in response to invading infectious bacterial and viral pathogens. The ability of the response of cellular mediated immunity for the recognition of the wide range of pathogens expressed as pathogen associated molecular patterns [2]. In most of the studies, it was exploited that the immunological involvement (humoral response) plays a vital role in the vaccine production; rarely the data was published [3].

Leptospirosis is a contemporary zoonotic disease mainly caused by *Leptospira interrogans* with a recent infection estimate of 1 million cases per year. This spirochetal bacterial infection is potentially life-threatening with increased mortality rate from 1 to 15% recent years [4, 5, 6]. In the first phase of leptospiral infection, there is a marginal elevation in polymorphonuclear neutrophils (PMN), further it get increased and considered as non specific acute sepsis and leptospiremia [7, 8, 9]. During gram negative bacterial and leptospiral infections, the implication of a major chemokine (interleukins) are observed in many cases that are induced by PMN and respond to cell activation that mediate innate immunity [5].

In case of acute phase protein (CRP) elevation, the immune responses damages multiorgan including liver, lungs, spleen, kidneys etc that are providing promising biomarker estimation for the prediction of the severity of the disease. To understand the importance and active role of immune response related to organ damage due to various infectious moieties, analysis the synthesis of inflammatory cytokines, chemokines and related adhesion molecules are relatively investigated in various cases, but scanty studies were recorded related to leptospirosis in order to understand the pathogenesis. A study highlighted the

induction and production of interleukins is delayed [10].

The C-reactive protein has been routinely identified as a major distinctive factor to rule out viral infections from bacterial; but few studies only suggestively defined the sensitivity and specificity. Based on the literature analyzed, the authors suggested that monitoring the changes in the leucocyte population and CRP associated with the acute to chronic bacterial infections with the host response to specific pathogens. The main aim of this investigation was to comparatively analyze the levels of CRP and neutrophils as earlier diagnostic markers of bacterial and viral infections.

MATERIALS AND METHODS

Study area and population

This study was conducted in three districts (Thanjavur, Tiruchirapalli and Pudukkottai) of central Tamilnadu. The patients who are attending the general outpatient department with fever with various etiology were included. This study was reviewed and approved by Institutional ethics committee (CMCH&RC/IEC-No: 158/ 26.11.2015). A total of 275 samples were processed to assess the infectious state of the subjects included and they were classified into three groups including 175 confirmed bacterial including leptospirosis, 45 viral infectious blood samples and 55 healthy control samples were included. Written informed consent was obtained from all patients and healthy individuals; the same was obtained from the parents if the subjects are children. The control groups were carefully analyzed for the absence of any infections, inflammatory diseases and immunological disorders. The patient groups were further subdivided into samples taken on day 1 to day 5 after initial symptoms of pyrexia of unknown origin (PUO). The socio-demographic details and basic clinical laboratory data which were collected from laboratory were impregnated in table 1.

Neutrophil isolation and characterization

The whole blood samples approximately 2ml drawn from all the patients and control groups were included in this study. If specific treatment were started, then the subjects were excluded from the study. All the collected blood samples were centrifuged for the separation of cells and plasma; thereby the plasma was stored and freeze temperature until further use. The red blood cells were lysed and the remaining cells were washed with phosphate buffered saline and neutrophils were isolated as described earlier [2, 11]. The separated neutrophils were used immediately for quantitatively determined by stained with monoclonal antibodies CD282/ TLR2-PE for 30 minutes at 4^{o}C, further fluorescence staining of neutrophils was measured by flow cytometer. The purity of the neutrophils were also measured and plotted.

Assay of cytokines

The levels of cytokines of IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-17 and TNF-α were measured using standard commercially available cytokine assay kit by following the manufacturer's instructions. If the cytokine levels are lower as described in the kit and are defined as lower limit of detection [2]. All the data were statistically analyzed and reports were interpreted as comparative descriptions and percentages.

RESULTS

Socio-demographic details

Among the total 275 eligible subjects included in this study, 180 (65.5%) were males and 95 (34.5%) were females. Out of them, 17 (6.2%) were children. Most of the patients included in this study are belonging to the age group of 21 – 40 years; and the same matched control groups were included. Most of the patients and control groups were exposed to animals either for occupation or as pet (54.5%). According to the analysis of literacy, most of them completed higher secondary school education (41.1%). The detailed demographic details of the subjects and control were interpreted in table 1. While interview all the subjects, many of them are unaware about the infectious diseases and their source of infection, later we provided some handful informations to them (Table 2).

Table 1: Demographic data of the subjects and control

Characteristics		Patients (n= 220)	Control (n=55)
Gender	Males (n=180)	140 (63.6)	40 (72.7)
	Females (n=95)	80 (36.4)	15 (27.3)
Age group (in years)	< 12	13 (5.9)	4 (7.3)
	13 – 20	11 (5.0)	7 (12.7)
	21 – 30	56 (25.5)	15 (27.4)
	31 – 40	67 (30.4)	12 (21.8)
	41 – 50	42 (19.1)	13 (23.6)
	51 – 60	22 (10.0)	3 (5.4)
	> 60	9 (4.1)	1 (1.8)
Residential status	Rural	185	38
	Urban	35	17
Occupational status	Agricultural labourers	71	13
	Domestic servant	14	11
	Semi skilled workers	17	4
	Petty business	10	2
	Government employee	36	9
	Private employee	32	5
	Student	13	4
	Drivers	12	2
	Hotel Staff	4	2
	Unemployed	7	1
	Unclassified	4	2
Education	Illiterate	42	24
	Primary level	76	11
	Secondary level	98	15
	Graduate	4	5
Animal contact	Yes	121	29
	No	99	26

[Figure in parenthesis denoted percentages]

Table 2: Awareness about the source of infections (n=275)

Infection	Yes	No	No response
Bacterial infections			
Anthrax	112 (40.7)	151 (54.9)	12 (4.4)
Brucellosis	46 (16.7)	205 (74.6)	24 (8.7)
Cholera	110 (40)	154 (56)	11 (4)
Helicobacter pylori infection	24 (8.7)	237 (86.2)	14 (5.1)
Leprosy	104 (37.8)	162 (58.9)	09 (3.3)
Leptospirosis	114 (41.5)	150 (54.5)	11 (4)
Pseudomonas infections	123 (44.7)	144 (52.4)	08 (2.9)
Shigellosis	56 (20.4)	202 (73.4)	17 (6.2)
Syphilis	71 (25.8)	183 (66.6)	21 (7.6)
Tetanus	94 (34.2)	173 (62.9)	08 (2.9)
Tuberculosis	115 (41.8)	149 (54.2)	11 (4)
Typhoid	116 (42.2)	156 (56.7)	03 (1.1)
Urinary tract infections	112 (40.7)	146 (53.1)	17 (6.2)
Viral infections			
Swine flu	94 (34.2)	158 (57.4)	23 (8.4)
Avian flu	92 (33.4)	172 (62.6)	11 (4)
HIV/AIDS	154 (56)	114 (41.5)	07 (2.5)
Hepatitis groups	23 (8.4)	242 (88)	10 (3.6)
Herpes simplex	21 (7.6)	251 (91.3)	03 (1.1)
NIPAH	145 (52.7)	123 (44.8)	07 (2.5)
Chicken pox	167 (60.7)	103 (37.5)	05 (1.8)
Chikungunya	91 (33.1)	173 (62.9)	11 (4)
Dengue	111 (40.4)	154 (56)	10 (3.6)
Rabies	114 (41.5)	155 (56.4)	06 (2.1)

[Figure in parenthesis denoted percentages]

Clinical laboratory data

Total white blood cell count

In patients with various bacterial infections, the total white blood cell counts were higher than the controls. Even after 5 days also, the increased WBC count is not decreased. Further, after 7 days of treatment, slowly the WBC gets reduced in count. Six out of 175 cases with bacterial infections, even after 7th also there is no much changes in the WBC reduction. In the case of serologically confirmed leptospiral cases, the WBC count were increased drastically and reduced only after 5 days of treatment with high dose of doxycycline. But in the case of viral infections, WBC is significantly higher than those of controls in the early period of infections (day one of post infection) and subsequently decreased after 3 days of post infection with treatment. Normal range of WBC in healthy adult is 5 to 12 X 10^3/ mL. The detailed comparative descriptions of WBC count among bacterial and viral infections, and healthy controls were impregnated in table 3.

Table 3: Comparative descriptions of WBC count among subjects and controls

Days after infection	Highest range observed (X 10^3/ mL	No. of subjects
Bacterial infections (n=175)		
Day 1	17.6	78 (44.6)
Day 3	17.5	95 (54.3)
Day 5	17.2	116 (66.3)
Day 7	13.1	169 (96.6)
Viral infections (n=45)		
Day 1	15.4	24 (53.3)
Day 3	14.0	29 (64.4)
Day 5	12.3	32 (71.1)
Day 7	11.7	43 (95.5)
Control (n=55)	***9.4 to 11.7***	***55 (100)***

[Figure in parenthesis denoted percentages]

Neutrophil count

In bacterial infections, the total neutrophils were measured higher than the controls. Even after 5 days also, the increased neutrophils are not decreased. After 7 days of treatment, slowly the neutrophils gets reduced. Nine out of 175 cases with bacterial infections, even after 7th also there is no much changes in the neutrophil reduction. In the case of serologically confirmed leptospiral cases, the neutrophils were increased drastically and reduced only after 5 days of treatment with high dose of doxycycline intravenously. But in the case of viral infections, neutrophils is significantly higher than those of controls in the early period of infections (day one of post infection) and subsequently decreased after 5 days of post infection with treatment. The detailed comparative descriptions of neutrophils among bacterial and viral infections, and healthy controls were impregnated in table 4.

Table 4: Comparative descriptions of neutrophils among subjects and controls

Days after infection	Highest range observed (X 10^3/ mL	No. of subjects
Bacterial infections (n=175)		
Day 1	9.6	94 (53.7)
Day 3	8.4	96 (54.8)
Day 5	8.0	111 (63.4)
Day 7	7.5	123 (70.3)
Viral infections (n=45)		
Day 1	7.2	12 (26.7)
Day 3	4.5	17 (37.8)
Day 5	3.2	29 (64.4)
Day 7	1.9	30 (66.7)
Control (n=55)	***2.7 to 7.0***	***55 (100)***

[Figure in parenthesis denoted percentages]

C-reactive protein

The level of CRP is get higher in the day 1 infections of leptospirosis (36mg/dL) and slowly decreased in after high dose of doxycycline therapy upto 9.6mg/dL, but there is no much reduction observed. For those cases, vitamin C (1000 mg/ day) and vitamin E (800 IU/ day) were administered for 10 days. This placebo reduced the CRP level from 9.6 to 3.5 mg /dL among leptospirosis cases. Other bacterial infections, no drastic elevation was observed. In the case of viral infections especially in dengue cases, the elevation of CRP was upto 53.4mg/dL and slowly reduced with supportive therapy. The detailed comparative descriptions of CRP levels among bacterial and viral infections, and healthy controls were impregnated in table 5.

Table 5: Comparative descriptions of CRP levels among subjects and controls

Days after infection	Highest range observed (mg/dL)	No. of subjects
Bacterial infections (n=175)		
Day 1	36	24 (13.7)
Day 3	29	23 (13.1)
Day 5	24	19 (10.8)
Day 7	9.6	18 (10.3)
Vitamin C & E supplements	3.5	17 (9.7)
Viral infections (n=45)		
Day 1	53.4	7 (15.5)
Day 3	46.2	5 (11.1)
Day 5	29.6	4 (8.9)
Day 7	8.4	2 (4.4)
Control (n=55)	***2.7 to 7.0***	***55 (100)***

[Figure in parenthesis denoted percentages]

Cytokine production in patients

In general all the infected patients are recorded with the elevated cytokines with variations among the types. On comparing with bacterial infected patients, the viral infected patients showed higher level of cytokines especially IL 17 and others were found significant. But in the case of bacterial infections alone, IL 1 and IL 2 levels were increased huge than other group of cytokines. IL 4, IL 8, IL 10, IL 12 and TNF α levels were significantly elevated in both bacterial and viral infections. There were significant differences in levels of IL 2, IL 8 and IL 10 on day one of infection between bacterial and viral infected groups.

The concentration of IL 8 on day 1 post viral infection was elevated that on day 3 and day 5 at the same way, IL 17 was significantly high on day 5 of post viral infection and lower than those of 1 and 3. The pictogram levels of different cytokines among bacterial and viral infections in different days (from 1 to 7 days) were depicted in table 6. In the case of leptospiral infections, the patients have the elevated cytokines of IL 2, IL 8 and IL 12 are elevated much than other bacterial infections (Figure 1).

Table 6: Cytokine levels of patients with bacterial and viral infections

Cytokine	Bacterial infections vs picogram levels of cytokines recorded				Viral infections vs picogram levels of cytokines recorded			
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4
IL 1	8	10	11	14	4	5	6	7
IL 2	17	12	10	10	1.5	2	2.5	4
IL 4	3	5	6	6	4.5	2	2	2
IL 5	3	1	1	1	3	2	2	1
IL 6	7	6	4	2	6	5	5	2
IL 8	4	4	3	2	6	5	5	3
IL 10	6	5	5	2	5	5	4	3
IL 12	6	6	6	3	6	6	5	3
IL 17	3	5	12	8	4	5	10	7
TNF α	46	48	54	51	43	26	22	20

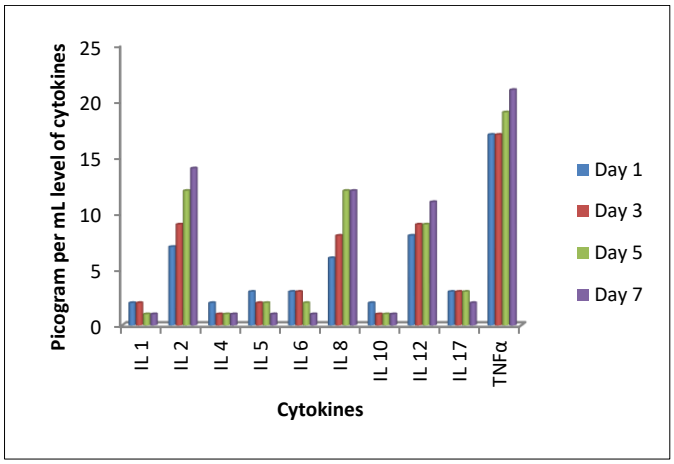

Figure 1: Determination of elevated cytokines in leptospirosis positive cases

DISCUSSION

From this investigation, it was found that the period of onset of infection or symptoms influences the expression levels of immunological and cytokinological get elevated upto the study days (7th day). The post infection and its related symptoms may play a major role in the cytokine expression; thus we may utilize this as a major biomarker to differentiate bacterial and viral infections with specificity and sensitivity.

We analyzed the levels of neutrophils from the patients included in this study and levels of cytokines in plasma samples. The review clearly suggested that the innate immune response to infection and the role of cytokines in relation to disease severity indicated that sera levels of cytokines were considerably higher in severe than in mild infections or healthy controls [2, 12]. The same was also observed in this current investigation thereby the elevated neutrophils and cytokines were observed.

The increased in neutrophil and cytokine levels are closely related to the time of post infection thereby it was observed higher when days increased. Other than TNF α, all others were elevated in progress when day increases in viral infections than bacterial. Eventhough the infection was treated with appropriate antibiotics, antiviral drugs and supportive therapy, the elevation of neutrophils and cytokines increased mainly due to the trigger of the immunological system. But in general, the TNF-α is subjected to analyze for the infectious print of viral infections and may be defined as the useful biomarker [13]. As TNF-α increases vascular permeability and killing of intercellular pathogens, further the upregulation of TNF-α in plasma may be persistent in normal to highly increase that depends on the infectious agents [14, 15].

In general, the patients with viral infections, cytokines level in the plasma is elevated during admission and further increased when days increased whereas TNF-α level is not increased. These data are considered as the useful biomarkers for viral infections. As TNF-α increases vascular permeability and killing of intracellular pathogens, where upregulation of TNF-α in plasma may be expected to persist in viral infections than bacterial [2].

The cytokines like IL 8, IL 12 and TNF-α are elevated more in bacterial infections even in the 7th day of the post infection whereas IL 17 and IL 10 are found in viral infections. In general, the role of cytokines is increased mainly due to migration and function of neutrophils. Additionally IL 6 influences the levels of CRP and IL 12 regulates gamma interferon production and the same was remaining in the host for 10 to 15 days. In general, on comparing with other interleukins, IL 6 elevated much in both bacterial and viral infections and remaining in the host for maximum of 15 days.

In specific, the IL 1, IL 2, IL 6, IL 8 and IL 17 levels in plasma may be able to distinguish bacterial and viral infections which may assist in ensuring appropriate choice of antibacterial and antiviral treatment. There was a markedly increased neutrophils are found high in circulation and tissues mainly in bacterial infections, whereas the role of antiviral defence is not much activated when the specific host system (organ) affected during viral infections [16]. The activated and successful functions of the neutrophils may be due to the specific viral infections like dengue fever.

During infection and mild to severe inflammation, the neutrophil levels in samples were elevated both in bacterial and viral infections which are found elevated compared to the control subjects. Neutrophils are defined as the first and predominant immune cell population to an affected site after viral infection, thereby antiviral defence role is mostly characterized and also have a pivotal role in the early stage of infection.

Mainly the antibacterial efficacy of neutrophils are highly determined by its mediate recognition of bacterial components responsible for infections and pathogenicity including endoflagellum in *Leptospira interrogans*, capsular polysaccharide in *Streptococcus pneumoniae*, enzymatic descriptions in *Staphylococcus* sp etc. In Swine flu (H_1N_1) cases, the H_2O_2 synthesis as a vital metabolite may be controlled by the neutrophils thereby the pathogenicitiy will be reduced.

The neutrophil polarization and plasticity in innate immunity has been encouraged to clear the pathogens from their targeted site, thus it was proved that neutrophil polarization influences the clearance of pathogens [17, 18]. In the present study also it was found that IL 4, IL 8, IL 10, IL 12 and TNF α levels are increased when days increases but infectious pathology reduced. Thus it was concluded that the neutrophil and cytokine levels in infection and inflammation clear the pathogen from the system at the earliest as the natural mechanism. Thus a supportive therapy to enhance the activity of neutrophils and cytokines may be provided for the patients in future.

CONCLUSION

Thus, the screening of concentration of neutrophils and cytokines in bacterial and viral infections may provide the fingerprinting for the determination of microbial infections. Also it was suggested to screen the patient for appropriate infections with clinical analysis and laboratory investigations at the earliest for the appropriate therapeutic interventions. Further inclusion of more patients to analyze this issue will help to address because the number of samples was small in the present study.

Conflict of Interest: No conflict of interest

Authors' contribution

Prabhusaran N - Data collection, results analysis and compilation

Prabhakar YK - Partial completion of the laboratory works

Pramila M - Partial completion of the laboratory works and result analysis

Naratajaseenivasan K - Result analysis and interpretations

Joseph Pushpa Innocent D - Data collection and overall result analysis

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