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Serum Levels of Copper in Human Leptospirosis Cases.

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ABSTRACT

Background: Copper deficiency can weaken immunity and increase the incidence of infections or infections may reduce the serum copper levels. **Objectives:** The present investigation assessed the serum level of copper in the cases of leptospirosis. **Methods:** The study involved 55 patients of whom had either icteric or non-icteric type of leptospirosis, 25 as other febrile and 25 as healthy controls. Serum copper was evaluated using commercially available kits. All the 55 patients are serologically confirmed for leptospirosis by using both ELISA and MAT. Twenty five age-matched febrile cases other than leptospirosis and twenty five healthy normal individuals were taken as control. The serum copper level was estimated by calorimetric method of Di-Br-PAESA. **Results:** The maximum and minimum age of patients was 54 and 18 years respectively; males dominated with 47 cases in this study. Out of 55 cases, 45 cases are considered as high risk groups of having minimum of 10 years of occupational exposure. There was a significant decrease of serum copper level among 55 leptospirosis confirmed cases (ranged from 26 to 63 µg/dl) when compared with controls (ranged from 114.12 to 126.32 µg/dl); whereas in febrile cases other than leptospirosis showed maximum of serum decrease upto 52.7 µg/dl. **Conclusion:** In all cases including non leptospirosis febrile cases also, the serum copper levels are significantly decreased after treatment with doxycycline and other antibiotics. This indicates that serum copper can be used as a biochemical marker for screening leptospirosis, as a valuable prognostic indicator for monitoring disease.

Keywords: Leptospirosis, febrile, serum, copper.

INTRODUCTION

Leptospirosis is the widespread zoonotic infection of global importance and recognised as re-emerging bacterial infections. In India, leptospirosis occurs in both rural and urban areas and in temperate and tropical climates.[1] It is an occupational hazard for people who work outdoors or with animals, such as farmers, rice mill workers, sewer workers, veterinarians, dairy workers, military personnel etc. The hot endemic pockets are five states and one union territory of India including Gujarat, Maharashtra, Kerala, Tamilnadu, Karnataka and Andaman and Nicobar Islands respectively.

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Humans get infection accidentally by their occupation and environmental exposure to infected animal's urine.[2] Initially it causes pyrexia, then liver involvement leads to jaundice. If not treated multiorgan dysfunction to multi organ failure observed. Mortality rate are low due to availability of antibiotics and other supportive drugs. In chronic cases, due to mutiorgan failure, the patient may die.[3]

It is underdiagnosed disease and mostly the physicians neglected this infection in their suspicion. In many literatures, the Scientist and Microbiologists suggested to suspect all fever cases as leptospirosis. But still the laboratory requests from clinical practice are scanty of sending samples for leptospirosis diagnosis. When the patients have chronic hepatospleenomegaly involvement with or without renal complications, then only the picture of leptospirosis comes to the clinical picture. Until

otherwise none of the pyrexia of unknown origin (PUO) is suspected with leptospirosis.[4,5]

Apart from microbiological investigations, the role of biochemical parameters may support to some extend to treat the patients appropriately.[6] Among them determination of serum urea, creatinine, urine complete are routine. The role of determination of microelements in leptospirosis cases is elucidated in larger prospective studies to inform public health interventions.[7] But the biochemical fingerprints including copper, iron and other macro and micro elements determination are measly and mainly used for research purpose only.

Leptospirosis is exacerbated by a weakened immune system. In general, copper and zinc are the important component of the immune system.[8,9] Cardiovascular pathology due to the deficiency of copper is well documented in animals and humans with various genetic diseases and also proved as a immunomodulators,[10] but the effect of copper status in infectious diseases needs elucidation. Studies highlighted the deficiency and copper in experimental animals showed impaired response.

There is a decreased phenomena in macrophage function, SOD activity of neutrophils, oxygen and cytokine production and inactive in antimicrobial due to copper deficiency, finally decreased in ceruloplasmin, an acute phase protein. But no report so far recorded in the demonstration of copper deficiency that impairs acquired immune function. Most studies suggested that copper deficiency affects specific immunity after extended periods of time.[11,12] In general, copper deficiency can weaken immunity and thus increase the incidence of infections. Based on this phenomena, an objective is determined to analyze the serum copper levels in serologically confirmed leptospirosis cases compared with other fever cases and healthy controls.

MATERIALS AND METHODS

The study was carried out in Ponniah Ramajayam Institute of Medical Sciences, Kancheepuram and Chennai Medical College Hospital and Research Centre, Tiruchirapalli during the period of January 2015 to December 2016. This study included 55 patients with leptospirosis, confirmed by clinical, cultural and serological examinations and who are admitted or attended in various clinical outpatient departments irrespective of age. Another age matched 25 other febrile cases (non reactive to leptospirosis) and 25 healthy control were included in this study.

This study was approved by the institutional ethical committee and written informed consent was obtained from all subjects participating in this study after explaining the nature of the study to them and confidentiality was maintained. The other confirmed febrile cases including classic, nosocomial, immune deficient and malignancy. Among 25 healthy controls, all are thoroughly determined for the absence of any diseases and no history of fever and other severe systemic infections minimum for the past 6 months.

The leptospirosis cases were confirmed by clinical manifestations interviewed during their outpatient visit and admission followed by culturing the blood in Ellinghausen-McCullough-Johnson-Harris (EMJH) semisolid medium and confirmed by dinger's ring and leptospires in dark field microscopy (DFM) (care must be taken for the exclusion of debris and other artifacts) and positive serology including genus specific Enzyme linked immunosorbent assay (ELISA) and serovar specific Microscopic agglutination test (MAT) whose titre value is reactive 1:80 and above. The major inclusion criteria of these cases are supported positive to minimum of 4 criteria. The other febrile cases were included based on the availability of the data in the case sheet that confirms the specific fever cases.

After obtained prior consent, 2ml of venous blood was collected in a sterile vial from the leptospirosis confirmed cases, and febrile and healthy controls. Lipidemic and hemolysed blood samples were rejected and discarded. Reagents used in this study for determining the serum copper levels were on analytic grade and organic solvents were redistilled before use. The estimation of serum copper was done based on reagent 4-(3,5-dibromo-2-pyridylazo)-N-ethyl-N-sulfo-propylaniline (Di-Br-PAESA) method. The stock solution of 0.02 mmol/L of Di-Br-PAESA (dissolve 1mg in 100mL acetate buffer) was prepared. The working copper color reagent is prepared by adding 1mL of 0.35mol/L ascorbic acid solution to 14mL of Di-Br-PAESA stock solution. After mixing serum, standard or distilled water for reagent blank sample of 0.1ml with reagent of 1.5ml, the mixture was incubated at 37^{o}C for 5 minutes, and then the absorbance was measured at 580nm in atomic absorption spectrophotometry (AAS). The normal serum copper level is ranged from 63.7 to 140.12µg/dL.[13] Then the copper concentrations were calculated from the calibration curve.

RESULTS

Age and sex matched case and controls were included in this study, thereby the detailed sociodemographic data including age, gender, occupation, animal contact and blood group were included. Further to all the subjects included, a detailed knowledge questionnaire was asked to determine the awareness about leptospirosis. The detailed data of the subjects were tabulated [Table 1].

The selection and inclusion of subjects who are confirmed with clinical manifestations and laboratory tests of the leptospirosis cases are

interpreted in table 2. In this study, the criteria for inclusion of subjects are supported positive to minimum of 4 criteria as mentioned in the materials and methods. Among the cases, 12 are excluded due to non supportive with 4 criteria. Thus among 67 cases, 55 were included.

Table 1: Sociodemographic description of the subjects included.

Category	Leptospirois positive cases (n=55)	Other febrile cases (n=25)	Healthy controls (n=25)
Age group (in years)			
Below 20	3 (5.5)	2 (8)	1 (4)
21 – 30	12 (21.8)	5 (20)	5 (20)
31 – 40	24 (43.6)	11 (44)	10 (40)
41 – 50	10 (18.2)	4 (16)	6 (24)
Above 50	6 (10.9)	3 (12)	3 (12)
Gender			
Male	47 (85.5)	20 (80)	19 (76)
Female	8 (14.5)	5 (20)	6 (24)
Occupation			
Farmers*	26 (47.3)	4 (16)	3 (12)
Sewer workers*	13 (23.7)	4 (16)	2 (8)
Butchers*	2 (3.6)	1 (4)	-
Mason*	2 (3.6)	-	2 (8)
Veterinarians*	2 (3.6)	-	1 (4)
Students	5 (9.1)	7 (28)	4 (16)
Professionals	3 (5.5)	4 (16)	6 (24)
Others	2 (3.6)	5 (20)	7 (28)
* All the subjects are having more than 10 years of the occupational experiences			
Blood group			
A positive	6 (10.9)	2 (8)	2 (8)
B positive	11 (20)	3 (12)	2 (8)
AB positive	8 (14.5)	4 (16)	5 (20)
O positive	25 (45.5)	14 (56)	15 (60)
A negative	1 (1.8)	1 (4)	-
B negative	-	-	-
AB negative	1 (1.8)	-	-
O negative	3 (5.5)	1 (4)	1 (4)

[Figure in parenthesis denoted percentages]

Table 2: Leptospirosis confirmed cases.

Criteria	Subjects supported (n=67)
Clinical symptoms + Culture with Dinger ring + Dark field microscopy + ELISA + MAT	17 (25.4)
Clinical symptoms + Culture with Dinger ring + Dark field microscopy + ELISA	14 (20.9)
Clinical symptoms + Culture with Dinger ring + Dark field microscopy + MAT	11 (16.4)
Clinical symptoms + Culture with Dinger ring + ELISA + MAT	13 (19.4)
Clinical symptoms + ELISA + MAT with very low titre	4 (6.0)
Clinical symptoms + ELISA	6 (9.0)
Clinical symptoms	2 (2.9)

[Figure in parenthesis denoted percentages]

Among the 55 MAT positive subjects, the serovar Grippotyphosa dominated followed by Australis and Icterohaemorrhagiae. The detailed description of mono, di and poly serovar in serum samples are depicted in [Figure 1].

The inclusion of various febrile cases as control with its percentages are classic (56%), nosocomial 20%); immune deficient (12%) and malignancy (12%), further the subtypes are depicted in [Table 3].

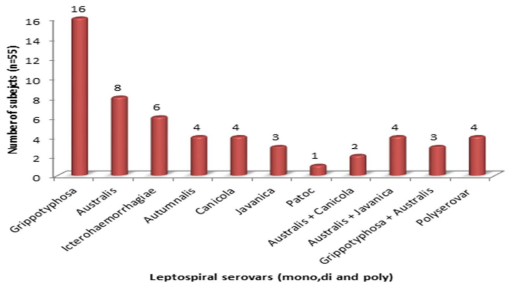

Figure 1: Serovar determination of positive MAT cases among leptospirosis

Table 3: Distribution of various febrile cases as control.

Febrile groups	Types	Number of subjects (n=25)	Percentage
Classic	Malaria	4	16
	Dengue	4	16
	Typhoid	3	12
	Chikungunya	3	12
Nosocomial	Drug induced	2	8
	Septic thrombophlebitis	1	4
	Sinusitis	2	8
Immune deficient	Tuberculosis	1	4
	Candidiasis	2	8
Malignancy	Non specific	3	12

The normal serum copper level is ranged from 63.7 to 140.12µg/dL. In this study, among the leptospirosis cases, a maximum of 27 cases supported the copper range of 46 to 55 µg/dL followed by 13 cases in 36 to 45 µg/dL. Only one sample showed 115 µg/dL of serum copper level [Table 4]. Further among other febrile cases, 14 samples supported to range of 64 to 140 µg/dL (normal range) followed by 4 samples in 56 to 63 µg/dL. All the healthy controls are recorded as the normal range of serum copper levels [Table 4].

Table 4: Distribution of serum copper ranges in leptospirosis, other febrile cases and healthy controls.

Serum copper range (µg/dL)	Leptospirosis cases (n=55)	Other febrile cases (n=25)	Healthy controls (n=25)
< 140	-	3 (12)	-
64 – 140 (normal level)	1 (1.8)	14(56)	25 (100)
56 - 63	7 (12.7)	4 (16)	-
46 - 55	27 (49.1)	4 (16)	-
36 - 45	13 (23.7)	-	-
26 - 35	6 (10.9)	-	-
> 25	1 (1.8)	-	-

[Figure in parenthesis denoted percentages]

Among the 55 serum samples that had detectable antibodies to leptospirosis, the serum copper levels

were also decreased in all samples except non pathogenic serovar Patoc showed the serum copper level of 129µg/dL. The serology positive against the serovar Autumnalis and polyserovar MAT reactive samples showed very less serum copper levels (26 to 35 µg/dL) followed by the serovar Icterohaemorrhagiae and diserovar MAT reactivity (Australis + Canicola and Australis + Javanica) (36 to 45 µg/dL). The detailed description of the decreased status of serum copper levels in various leptospiral serovars are depicted in [Table 5].

Table 5: Comparativeness of MAT positivity and serum copper levels.

Leptospiral serovars	Number of samples supported (n=55)	Serum copper levels (µg/dL)
Mono-serovar MAT reactivity		
Grippotyphosa	16 (29.0)	46 to 55
Australis	8 (14.5)	46 to 55
Icterohaemorrhagiae	6 (10.9)	36 to 45
Autumnalis	4 (7.3)	26 to 35
Canicola	4 (7.3)	56 to 63
Javanica	3 (5.5)	56 to 63
Patoc	1 (1.8)	129
Poly-serovar MAT reactivity		
Australis + Canicola	2 (3.6)	36 to 45
Australis + Javanica	4 (7.3)	36 to 45
Grippotyphosa + Australis	3 (5.5)	46 to 55
Australis + Javanica + Grippotyphosa + Icterohaemorrhagiae	4 (7.3)	26 to 35

[Figure in parenthesis denoted percentages]

There is no such high decrease in the serum copper level in other febrile cases; but observable reduction was recorded. In malaria and dengue cases, decrease in serum copper level was observed than normal range (63.7 to 140.12µg/dL). In non specific malignancy cases, there is an elevation in the serum copper levels from 196 to 204µg/dL. The various ranges among other febrile cases were impregnated in Table 6.

Table 6: Comparative analysis of serum copper levels among febrile cases other than leptospirosis.

Other febrile cases	Number of subjects (n=25)	Serum copper levels (µg/dL)
Malaria	4	52 to 59
Dengue	4	54 to 65
Typhoid	3	74 to 106
Chikungunya	3	64 to 94
Drug induced	2	96 to 129
Septic thrombophlebitis	1	112
Sinusitis	2	96 to 126
Tuberculosis	1	121
Candidiasis	2	106 to 114
Non specific malignancy	3	196 to 204

DISCUSSION

The role of copper in the leptospiral growth under *in vitro* studies proved that micronutrients including copper extend the spirochetal structure in the principle of maintenance of long chain fatty acid. Due to the accumulation of copper in the human and animal systems during the leptospiral infection leads to deposition of copper molecules in the liver and such condition is defined as copper storage hepatopathy.[14] In cancer cases, a positive correlation between serum copper level and grades of cancer were noticed leads to linear progression of copper level with increasing stages of the disease.[15] The same was also identified in this study by elevation of serum copper level in malignancy cases.

In general, immune system required copper to perform several functions including interleukin 2, T cell proliferation, maintaining neutrophil concentrations etc.[16,17] Development of markers sensitive to marginal copper status is essential before conclusions can be drawn concerning the risks of long term intake of suboptimal dietery copper.[18] Some studies highlighted that the diagnosis of marginal copper deficiency may reduce the immune status;[19] thus we suggested to the clinicians to supplement the leptospirosis therapy with copper or advice to have copper diets.

Copper deficiency can also be seen in individuals having serious digestive disorders that impair nutrient absorption including Crohn's disease; further absorption of copper can be impaired from very high intakes of iron or zinc usually from supplements. A number of trace elements, especially those having oxidative properties such as copper, iron and zinc, play an important role in infectious disease progression.[20] Among these important trace elements, the present study estimated the levels of copper, whereas serum copper levels have been studied and associated with leptospirosis severity. However other studies have indicated that copper supplementation resulted in variable immune responses depending on the class of immune cell being studies, as well as the source and concentration of supplemental copper.[8,21]

The increase in copper level among the cases might result from increased liver production of ceruloplasmin as an inflammatory response to the immunocompetent cases including cancer.[8,22,23] Controversially, the authors suggested the phenomena that decrease in copper level among the leptospirosis cases may decrease the liver production of ceruloplasmin. Further, measurement of serum ceruloplasmin among reactive leptospirosis samples may help to some extend to determine the liver functioning in order to treat the cases and protect the liver in the early stage of infection and inflammation itself.

Changes in the levels of micronutrients are influencing oxidative stress during infections. Copper acts as a cofactor of antioxidant enzymes to protect the body from the oxidative stress response under normal circumstances. In chronic viral infections, the anti-oxidative capacity will be

outdated and accumulation of toxic lipid peroxidation products in the cells.[24]

CONCLUSION

Although normally bound to proteins, copper may be released and become free to catalyze the formation of free radicals that have the capacity to initiate an oxidative damage and interfere with cellular events.[25] So, it is mandatory to maintain the stable level of serum copper for protecting the cell from free radical toxins. Thus this study may be the initiative to include and interpret biochemical parameters for the diagnosis of leptospirosis and other infectious diseases.

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