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INVESTIGATION OF RELATIONSHIP BETWEEN SERUM C-REACTIVE PROTEIN (CRP) LEVEL AND THE RATE OF MICROBIAL INFECTION IN "COPD" PATIENT

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ABSTRACT

The present study aimed to investigate the relationship between the serum C-reactive protein (CRP) level and the rate of microbial infection in chronic obstructive pulmonary disease (COPD) patient. From November 2012 to October 2013, 138 serum and respiratory sample collected from stable COPD patient of private hospital south Tamilnadu, India. Serum CRP level measured by Immunoturbidity method and sputum sample were collected for culture. Among 138, the mean value of CRP in culture positive group (75 subject) was 148.94 (range : 50 mg/L to 250 mg /L) and SD 56, while in culture negative group (63 subjects) the mean value was 11.22 mg/L (range: 6- 20 in 40 subject) and SD was 4.64 and the mean value in 23 subject was 36.69 mg/L (range: >20 – 50mg/L in 23 subject) and SD was 9.20 in culture negative group. Statistical analysis, the mean value of CRP was significantly higher in culture positive patient (CRP level >50mg/L). In culture positive group the following organism were isolated with 54 % (14 Klebsiella (10%),13 Pseudomonas (9%), 1 Acinetobacter (1%), 3 E.coli (2%), 25 Haemophilus influenza(18%), 2 Staphylococcus aureus (1%), 3 Streptococcus pneumonia (2%), 11 candida species (8%), 3 Filamentous fungi (2%) and 63 recorded culture negative with 46% and 8 TB positive with 5%. The predominant bacterial isolate was Haemophilus influenza. The circulating level of CRP significantly elevated in culture positive stable COPD patient than culture negative patient. CRP value from > 6 to 50, in culture negative group those were had symptoms of chronic cough, chills and mild grade fever. It revealed, in COPD patient the CRP value was

above the normal range (>6 mg/L) even if culture negative cases it may be due to viral or intra nuclear infection.

KEYWORDS: Chronic Obstructive Pulmonary Disease, C-reactive protein, Bacterial infection.

INTRODUCTION

C-reactive protein (CRP) is an acute phase inflammatory response of protein synthesized predominantly by the hepatocytes in response to tissue damage or inflammation and a major component of the body ‘s innate deference mechanism. It reacts promptly without specificity or memory upon exposure to different types of inflammatory stimuli. The C-RP has an affinity to carbohydrate (C) antigen on the cell wall of bacteria and fungi, hence the name C-reactive protein. The acute phase response to comprises the changes in the serum protein profile during the inflammation process. Generally CRP in the blood in very low concentration (<1mg/L), the normal range of CRP 6 mg/L, but during inflammatory process, this concentration increases significantly. More than 20 epidemiological studies have demonstrated that low concentrations of CRP can be a marker of low level of infection and inflammation.[1]

Chronic obstructive pulmonary disease (COPD) encompasses several conditions (airflow obstruction, chronic bronchitis, bronchiolitis or small airways disease and emphysema. Patients with chronic bronchitis are more susceptible to bacterial bronchial infections than those at the emphysema or asthma end of the spectrum. Mucus hyper secretion, which is the hallmark of chronic bronchitis, is particularly associated with mortality from an infectious cause.[2]

Bacterial infection is only one of numerous stimuli causing inflammation in COPD and evidence is lacking that increased inflammation due to bacterial infection alone leads to progression of airflow obstruction. However, when infective exacerbation occurs, they have a major impact on the patient overall health status, which may take 6 weeks or more to recover and infection can lead to serious complications requiring hospitalization.[3]

The bacterial pathogen most commonly isolated from sputum during acute exacerbation of COPD are nontypable Haemophilus inflenzae, Moraxella catarhalis, and Streptococcus pneumonia.[4]

It is important to note that all studies to date of bacterial causation of exacerbations have used a single culture of sputum at the time of presentation and standard or semi quantitative culture techniques. No study has studied multiple sputum samples from each exacerbation or applied more sensitive techniques such as polymerase chain reaction for bacterial antigens to sputum samples. It is quite possible that such studies may reveal that a larger proportion of exacerbations has a bacterial origin than the current data suggest.[5]

Younger patient with COPD generally have a lower mortality rate unless they also have alpha$_1$ antitrypsin deficiency, a rare genetic abnormality that causes pan lobular emphysema in younger adult.[6] The approximate mortality at 1 yr and 10 yrs is 30% and 95%, respectively, although longitudinal studies have shown that some patients survive for many years beyond the average.[7]

Numerous studies performed in recent years provide overwhelming evidence of COPD as a condition characterized by an abnormal inflammatory response beyond the lungs with evidence of low –grade systemic inflammation which causes systemic manifestation such as weight loss, skeletal muscle dysfunction, an increased risk of cardiovascular disease, osteoporosis and depression, among others.[8]

Blood markers, such as IL-6, CRP and fibrinogen have attracted interest during recent years, and further studies in this area will probably increase the understanding of system manifestation in COPD (31). One of the inflammatory markers which is recently is increasingly evaluated in COPD patient is C-reactive protein.[9]

The aim of this study to evaluate the prevalence of microbial infection in stable COPD patient and estimation of the level of CRP in patient with COPD and study the relationship between the CRP levels and bacterial infection in COPD patient.

MATERIAL AND METHOD

Sample collection

The Blood samples were collected by veni puncture by using sterile, disposable syringe and needle. 2-3ml of blood was collected and immediately put in plain disposable test tube and allowed to clot. Sputum sample were collected in sterile container. The collected samples were aseptically transported to the laboratory. All sputum specimen were cultured on Blood agar, Chocolate agar and MacConkey agar medium under aseptic condition. All cultured

plates incubate aerobically at 37^0 C overnight, and then observed for the presence of bacterial growth.[10]

Measurement of C-reactive protein level

The Blood samples were collected by veni puncture by using sterile, disposable syringe and needle. 2-3ml of blood was collected in BD vacutainer and allowed to clot. The collected blood samples were aseptically transported to the laboratory within 24 hours. The clotted blood was centrifuged at 850 to 1000 g form 10 minutes to separate serum. Then the serum was transferred and stored in a small plastic vials. The separated serum samples were either tested immediately or were stored at 2-8C in refrigerator for maximum of 48 hours before testing.

The Commercially available CRP kit from SPINREACT COMPAY, SPAIN was used in this study. The manufacturer's instructions given along with the kit were followed. The -CRP kit is a quantitative turbid metric test for the measurement of low level of C-reactive protein (C-RP) in human serum. Latex particle coated with specific goat IgG anti-human C-RP are agglutinated when they mixed with serum samples containing C-reactive protein. The agglutination caused on absorbance change, depending on the CRP concentration of the patient's samples.

The C-RP contents in the sampling quantified by comparison from a calibrator of known CRP concentration. Reagents are diluents (Tris buffer 20 mmol/L, pH 8.2, sodium azide 0.95g/L), Latex particles coated with goat IgG anti-human C-RP, CRP calibrator-Human serum, CRP concentration was 10.5 mg /L stated on the vial label. The sensitivity of the assay and the target value of the calibrator have been standardized against the reference material CRM 470/PPHS (Institute for Reference materials and Measurement, IRMM). The latex vial was gently shaken before use. 1ml of Latex reagent was taken and mixed with 14ml of diluents.

The CRP calibrator was reconstituted with 2.0ml of distilled water. Before using the preparation was mixed and brought to room temperature for 10 minutes. CRP calibrator dilution were prepared by using sodium chloride 9 gm/L as diluents. The concentration of the CRP calibrator were multiplied with the corresponding factor values were given in the **Table 1.** Vital 21 chemistry analyzer was used to quantify (CRP) C-reactive protein level of the blood samples.

The working reagent, samples, flow cell of photometer was brought to 37 ^{o}c before the work was started. The assay condition used are Wave length 546 nm (530-550), temperature 37^{o} C . Using distilled water the instrument was adjusted to zero. 1ml of working reagent and 10 micro liter of serum were pipette out into a curvets. The working reagent and the serum sample were mixed and read the absorbance immediately (A1) and after 4 minutes (A2) of the sample addition. Calculate the absorbance difference (A2-A1) of each point of the calibration curve and plot the value obtained against the CRP concentration of each of calibrator dilution. C-RP concentration in the sample is calculated by interpolation of its (A2-A1) in the calibration curve.

Table 1 CRP immunoturbidmetry intra assay-precision

Assay no	Sample 1 (Low value) mg/l	Sample 2 (High value) mg/l
1	1.51	8.03
2	1.45	7.97
3	1.54	8.10
4	1.49	7.92
5	1.43	7.89

Identification of organism

The organism was identified based on the colony morphology on the culture media and the Biochemical tests according to Bergey's manual.[11]

Microscopic examination

Z/n stain/ gram stain

The sputum specimen were smeared and stained with z/n stain to detect the acid fast bacilli. The isolates were stained by Simple stain and Gram stain, wet mount preparation to detect their motility and gram's reaction, shape and their arrangement.[12]

Growth on media

MacConkey used for isolation and cultivation of lactose fermenting and non-lactose fermenting bacteria. Blood agar also used to cultivation of Gram negative bacteria and used to isolate Staphylococcus aureus and Streptococcus species, Candida species. Chocolate agar used to isolate Haemophilus influenae.[13]

Mannitol salt agar

The plates were streaked from pure colony of tested bacteria and then incubated for 24hrs at 37 ^{0} C. This media used for selective isolation and cultivation of Staphylococcus aureus based on mannitol fermentation.[14]

Biochemical tests

Catalase tests: A colony of tested bacteria was mixed with 3% H_2O_2 on the slide and positive results were indicated by air bubbles formation.[15]

Coagulase test

0.5 ml of citrated rabbit plasma (diluted 1 in 5 with saline) mixed with 5 drops (250 µl) of overnight broth culture or small amount of the colony growth of Staphylococcus aureus and Incubated at 37^0 C for 4 hours. A tube of plasma mixed with sterile broth was included as a control. Formation of clots in 1-4 hrs indicates a positive test. If no clot is observed at that time, reincubate the tube at room temperature and read again after 18 hours.[16]

Oxidase

Filter paper moistened with freshly prepared 1% oxidase reagent , a colony of tested of bacteria smeared on the filter paper, and positive results were indicated by development of deep blue color at the site of colonies smeared within 10 seconds. Negative result the colour of the colonies remain unchanged.[17]

Indole

Tryptophan broth inoculated with the tested bacteria and incubated at37^0 C for 24 to 48 hrs. 0.2 ml of Kovac's reagent added to the 0.5 ml culture broth. Formation of red-violet ring within minutes indicates a positive test. Development of yellow ring indicate negative test.[18]

Methyl red and Voges Proskaur test

A colony of tested bacteria inoculate in MR-VP medium and incubated at37^0 C for 24 to 48 hrs. 0.04 % methyl red added to the broth of culture. Development of stable red color indicate MR positive. Negative no color change. 5% naphthol and 40 % KOH (VP reagent added to the VP medium. Development of pink colour within 2-5 minutes indicate positive test. No pink color within 15 minutes indicate negative test. The test should not read after for over 1 hour it leads false positive interpretation.[19]

Citrate utilization

A colony of tested bacteria inoculate in simmon' citrate medium and incubated at37^0 C for 24 to 48 hrs. Development of deep blue colour with in 24 to 48 hours of incubation indicate positive test. Negative test indicated by no colour change of the citrate medium.[20]

Triple sugar iron agar

A colony of tested bacteria inoculate in TSI (Triple sugar iron agar) medium and incubated at37^0 C for 18 to 24 hrs. Observerd the colour chage in the slant and butt and also observed the development of black precipitation indicate H_2S production. Alkaline slant and alkaline butt (K/K) indicate no carbohydrate fermentation and non-fermentor. Alkaline slant and acidic butt (K/A) indicate glucose fermented and lactose, sucrose was not fermented it showed the tested bacteria were non-lactose fermentor. Acidic slant /acidic butt (A/A) all sugars (lactose, glucose and sucrose) were fermented, it indicate lactose fermenting bacteria.[21]

Satellitism test to identify Haemophilus influenzae

Suspected colonies of *Haemophilus* colonies mixed in 2 ml of sterile physiological saline (or sterile peptone water). Inoculated the organism suspension on a plate of blood agar by sterile swab. . Streaked a pure culture of *S. aureus* across. The inoculated plates were incubated in a carbon dioxide enriched atmosphere at 35 to 37^{o}C for 18-24 hours. Examined the culture plates for growth and satellite colonies. The colonies near the column of *S. aureus* growth it indicate satellite colonies. The satellites colonies on both blood agar the organism probably a *Haemophilus* species.[22]

RESULT

In this Study one hundred and thirty eight sputum and serum specimen were collected from stable COPD outpatient of private hospital during the period from November 2012 to October 2013 in the south Tamilnadu, India. Out of 138 specimen, 75 recorded a culture positive with a percentage of 54 % and 63 recorded culture negative with a percentage 46 % and 8 patient TB positive with a percentage of 5 %. The age of the patient rages from 20 to 70 years. According to the age,10 sample culture positive, 19 culture negative in <30 age group and 65 sample culture positive, 44 culture negative in >30 age group of COPD patient.[Table 2]

Among 138, the mean value of CRP in culture positive group (75 subject) was 148.94 (range: 50 mg/L to 250 mg /L) and SD 56, while in culture negative group (63 subjects) the mean value was 11.22 mg/L (range: 6- 20 in 40 subject) and SD was 4.64 and the mean value in 23 subject was 36.69 mg/L (range: >20 – 50mg/L in 23 subject) and SD was 9.20 in culture negative group.[Table 3-5, Fig 2-4) .

In culture positive group the following organism were isolated with 54 % (14 Klebsiella (10%),13 Pseudomonas (9%), 1 Acinetobacter (1%), 3 E.coli (2%), 25 Haemophilus influenza(18%), 2 Staphylococcus aureus (1%), 3 Streptococcus pneumonia (2%), 11 candida species (8%), 3 Filamentous fungi (2%) and 63 recorded culture negative with 46% and 8 TB positive with 5%. [Table 6, Fig 5] The predominant bacterial isolate was Haemophilus influenza.

Statistical analysis, the mean value of CRP was significantly higher in culture positive patient (CRP level >50mg/L). The circulating level of CRP significantly elevated in culture positive stable COPD patient than culture negative patient. CRP value from > 6 to 50, in culture negative group those were had symptoms of chronic cough, chills and mild grade fever. It revealed, in COPD patient the CRP value was above the normal range (>6 mg/L) even if culture negative cases it may be due to viral or intra nuclear infection.

Table 2 Distribution of study sample and rate of isolation according to age

Age group	<30	>30	Total
No of sample tested	29	109	138
Culture positive	10	65	75
Culture negative	19	44	63

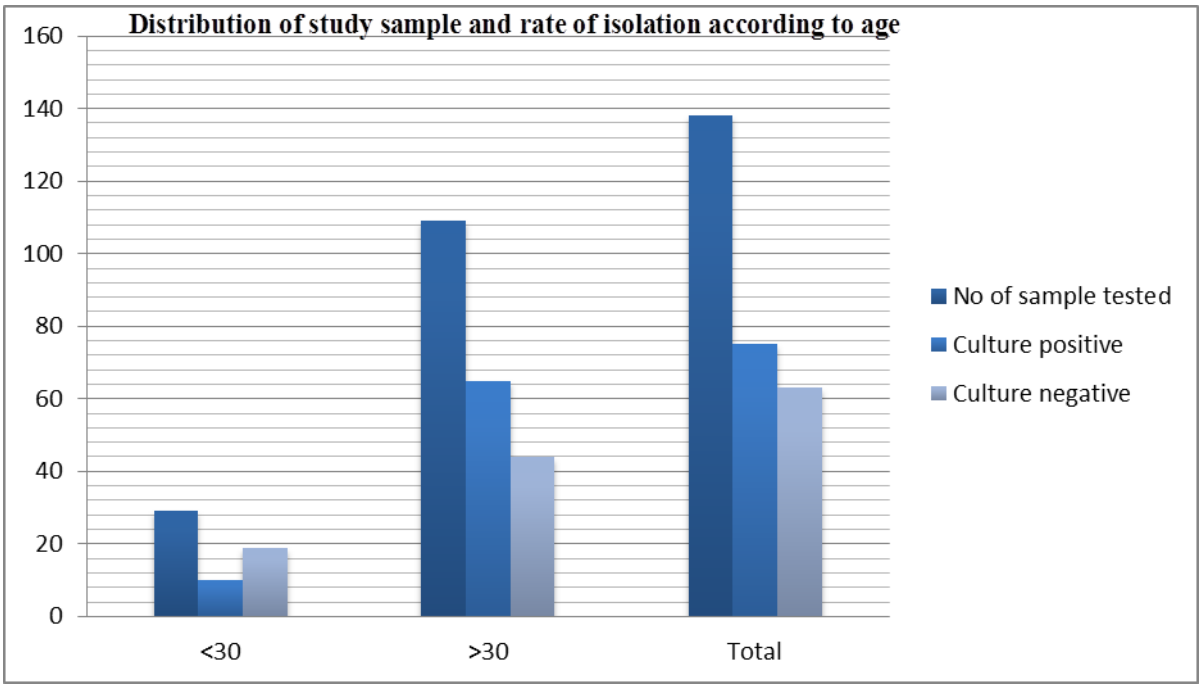


Table 3 Distribution of CRP level in 40 patient (<20 mg/L)

CRP level mg/l	No of sample
< 6	5
6 to 10	15
11 to 20	20
Mean value	11.23
Mode	8.90
SD	4.64

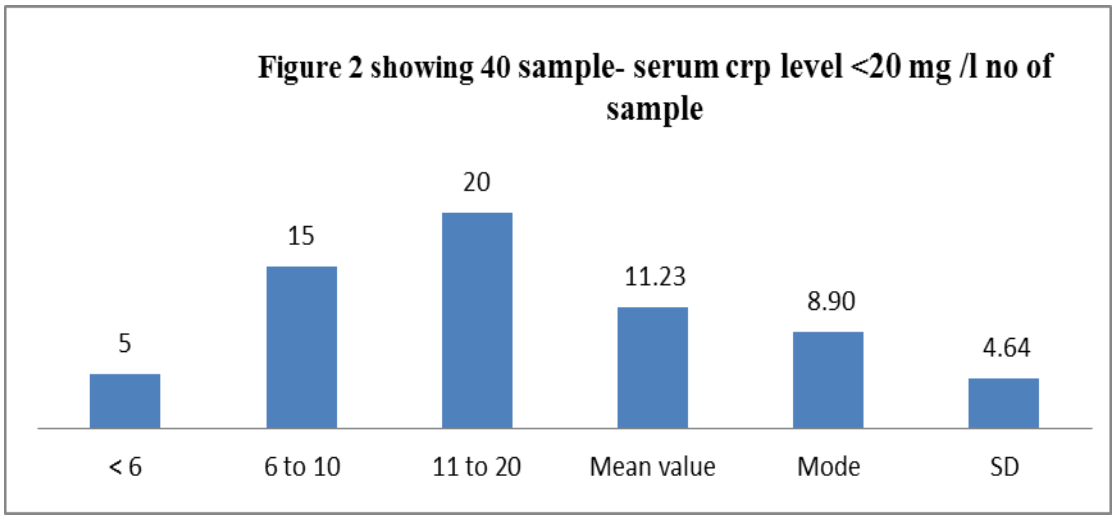


Table 4 distribution of CRP level of 23 patient >20 to 50 mg /l

CRP LELVEL mg/L	No of sample
21 to 30	5
31 to 40	10
41 to 50	8
Mean value	36.69
Mode	48.9
SD	9.20

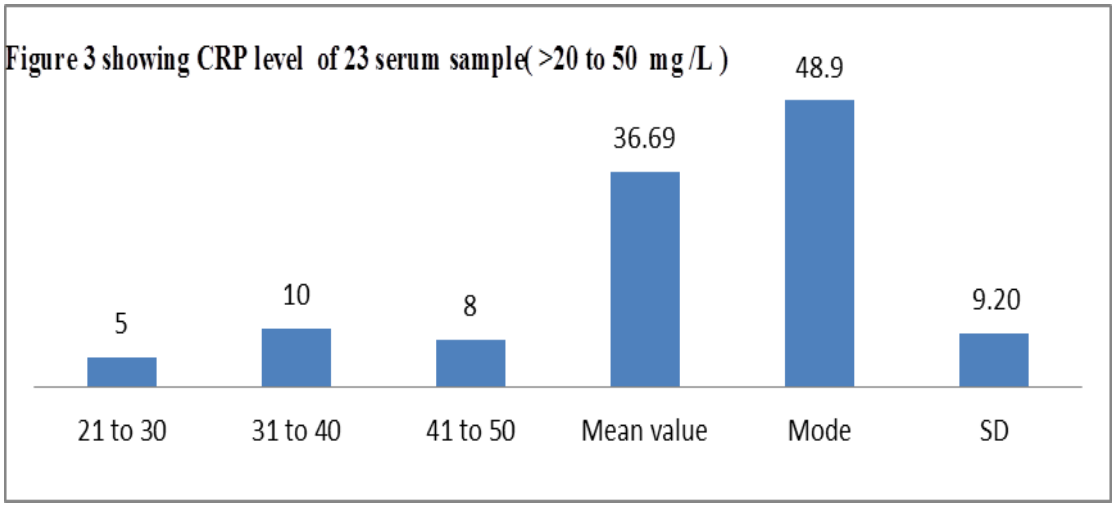


Table 5 Distribution of CRP level in 75 patient >50 to 250 mg /L

CRP lelvel mg/l	No of sample
50 to 70	3
71 to 90	12
91 to 110	9
111 to 130	7
131 to 150	8
151 to 170	6
171 to 190	8
191 to 210	8
211 to 230	8
231 to 250	6
Mean value	148.9453
Mode	0
SD	56

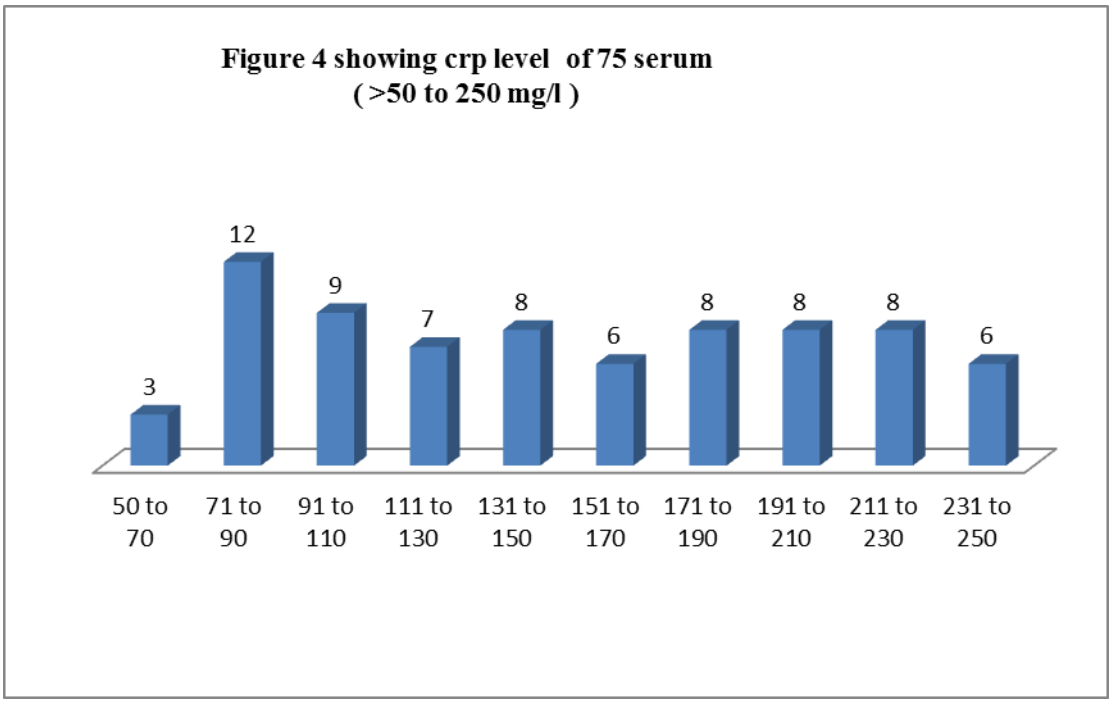
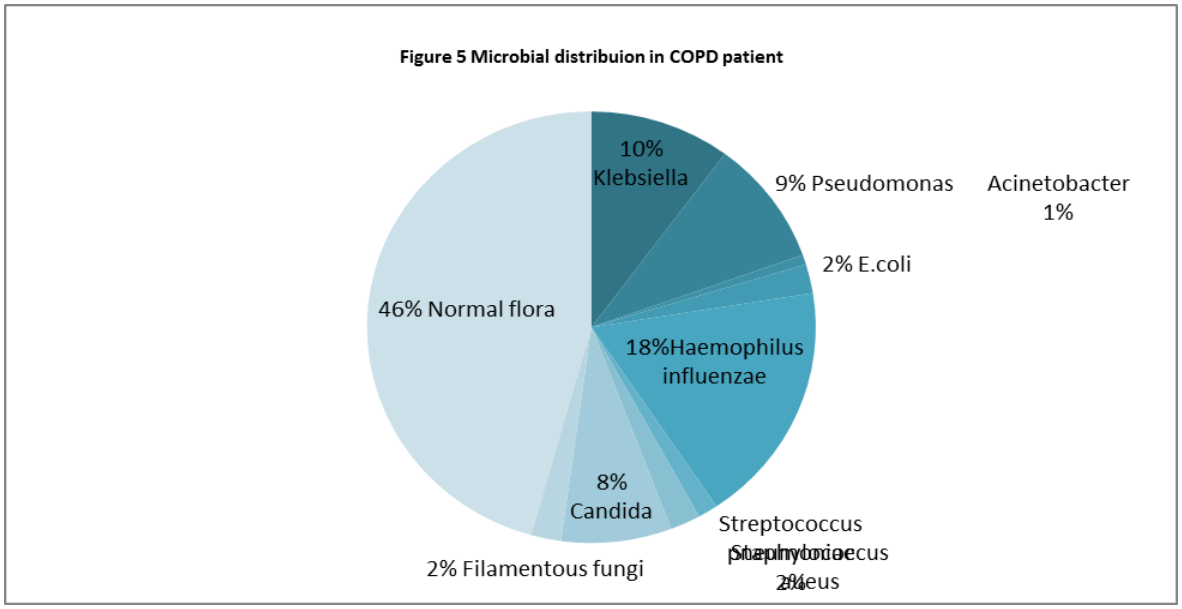


Table 6 Comparison of culture positive and negative groups for value of CRP by statistical analysis

Group	Sample size	Mean	Mode	Standard deviation
Culture Negative (CRP level <20)	40	11.23	8.9	4.64
Culture Negative (CRP level 20 to 50)	23	36.69	48.9	9.2
Culture Positive (CRP level >50 to 250)	75	148.94	0	56

Table 7 Distribution of CRP values in patients of COPD with culture positive

Value of CRP mg/L	No of copd patient(n=75)	Organism isolated
50 to 70	3	Candida
71 to 90	12	Filamentous fungi
91 to 110	9	Acinetobacter
111 to 130	7	E.coli
131 to 150	8	Haemophilus influenzae
151 to 170	6	Staphylococcus aueus
171 to 190	8	Streptococcus pneumoniae
191 to 210	6	Klebsiella
211 to 230	6	Pseudomonas aeruginosa
231 to 250	6	Klebsiella
211 to 230	2	Pseudomonas aeruginosa
231 to 250	2	Candida+ TB Positive



DISCUSSION

CRP, an acute phase protein, has been widely used clinically for many years as a diagnostic tool for infection identification. (16 d. The level determined exclusively by its rate of synthesis, which reflects the presence and extent of disease activity.17). CRP is an important biomarker in COPD, indirect evidence of infection and inflammation. It is a better indicator of infection. It is an early maker of the exacerbation and also beneficial in assessing efficacy of the treatment. Our study showed that the circulating level of CRP was significantly elevated in patient with COPD.

Mean value of CRP in culture positive group (75 subject) was 148.94 (range : 50 mg/L to 250 mg /L) and SD 56, while in culture negative group the mean value was 11.22 mg/L (

range: 6- 20 in 40 subject) and SD was 4.64 and the mean value in 23 subject was 36.69 mg/L (range: >20 – 50mg/L in 23 subject) and SD was 9.20 in culture negative group. When compared with culture negative by independent samples t- tes , the mean value of CRP was significantly higher in culture positive patient with COPD.

In our findings out of 138 specimen, 75 recorded a culture positive with a percentage of 54 % and 63 were recorded as culture negative with a percentage of 46 % and 8 patient TB positive with a percentage of 5 %. The age of the patient ranges from 20 to 70 years. Out of 138 patient, 109 were > 30 years, 29 were < 30 years. The result showed that the age group > 40 years was more number of bacteria isolated from 65 patient. Fifty four percentage (54 %) of specimen showed significant growth, 46 % recorded no growth. Among 75 isolates, 14 Klebsiella sps (10%), 13 Pseudomonas sps (9%), 1 Acinetobacter (1%), 3 E.coli(2%), 25 Haemophilus influenza (18%), 2 Staphylococcus aureus (1MRSA) (1%), 3 Streptococcus pneumonia (2%), 11 candida species (8 %), 3 Filamentous fungi (2%) 63 normal flora (46 %) were isolated.

Babu et al., demonstrated a higher value of CRP in pneumonia than in children with bronchiolitis or acute bronchitis and a fall in CRP provided an earliest clue to therapeutic response much before a fall in temperature, respiratory rate or ESR.

Morely and Kushner also reported that the significantly higher CRP in 9 patients with acute bacterial pneumonia in comparison with 9 patients with acute bronchitis. A retrospective observation on uncomplicated community acquired pneumonia involving 40 patients of pneumonia and 20 patients of COPD had demonstrated that it is possible to distinguish between pneumonia and infective bronchitis without pneumonia on the basis of serum CRP levels (5) Similarly, to assess the usefulness of serum C-reactive protein in the diagnosis and treatment approach of patients with community-acquired pneumonia, Spanish researchers carried out population-based case-control study in a mixed residential-industrial urban area of 74,368 adult inhabitants in Barcelona from 1993 to 1958.

The predominant bacterial species isolated from the stable COPD patient are Haemophilus influenza, Klebsiella sps, Pseudomonas aeruginosa, Pseudomonas fluorescens and candida species. The circulating level of inflammatory marker CRP are significantly elevated in culture positive stable COPD patient than culture negative patient.

CONCLUSION

In conclusion, Haemophilus influenza was found to predominant bacterial isolated in stable COPD patient. CRP is an important biomarker in COPD and indirect evidence of infection and inflammation. It is a better indicator of infection. It is an early marker of the exacerbation and also beneficial in assessing efficacy of the treatment. Our study showed that the circulating level of CRP was significantly higher in culture positive COPD patient (range from >50 to 250 mg/L) than culture negative COPD (range from > 6 to 50) and also it revealed that in COPD patient the CRP value was above the normal range (>6 mg/L) even if culture negative cases it may be due to viral or intra nuclear infection.

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