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The analysis of anaphylatoxin C5a with pneumonia patients in Rewa Region

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Abstract
The present paper deals the analysis of C5a with Pneumonia patients in Rewa region. For this work the blood sample of 240 pneumonic patients and 240 controls have been collected. The results of the C5a analysis indicated that C5a are found in normal blood level in healthy controls. Average value of blood C5a concentration was seen 23.5 ± 1.31 ng/ml in pneumonic patients and 5.7 ± 0.86 ng/ml in healthy controls. The median value of C5a blood concentration was found 90 pg/ml and 40 pg/ml. Standard error of the mean was found 8 pg/ml and 4 pg/ml respectively for patients and control. The value of t-test of the differences of C5a blood concentration between pneumonic patients and healthy control was found 173.59 with 998 degree of freedom which is statistically significant at the level of P <0.0001. Limit of C5a blood concentration in the patient was 3-28 ng/ml and 4-7 ng/ml was found in the control group.

Keywords: Pneumonia, Anaphylatoxin C5a

Introduction
Respiratory infections have become a common problem on these days. With thousands of people dying each year in India and around 4 million deaths worldwide, are attributed to pneumonia alone. With an increase in pollution and use of harmful airborne chemicals, it's becoming increasingly difficult to bring these numbers down. Pneumonia is an infection of respiratory organs within which the lung tissue of an infected person is crammed with fluid or pus. Individuals affected by this condition show symptoms of shortness of breath, fever, chills, chest and abdominal pain, presence of brown, yellow or green colored phlegm and cough (Grotto *et al.* 2003) [1]. Pneumonia is classified as: community-acquired, hospital-acquired and ventilator-associated occurring in people (individuals with weakened immune system). Community-acquired pneumonia is one in all the foremost common infectious disease requiring medical aid, and it's the third leading explanation for death worldwide (Fine *et al.* 1996) [3]. To protect kids from pneumonia, it is important to promote breastfeeding, hand laundry and to reduce indoor air pollution, stopping pneumonia with vaccinations, treating pneumonia and ensuring that each sick kid has access to the proper quite care, either from a community-based medical expert or during a sanatorium if the sickness is severe to get the antibiotics and oxygen (Brown and Roberts, 2004) [2].

Most proteins and glycoproteins that constitute the complement system are synthesized by hepatocytes. But significant amounts are also produced by tissue macrophages, hemoglobin, and epithelial cells of the genital system and gastrointestinal tract. The three pathways of activation produce all conjugal versions of protease C3-convertases. The classical complement pathway typically requires antigen-antibody complexes for activation (specific immune response), whereas the alternative pathway can be activated by activating 3 component (C3) hydrolysis, foreign materials, pathogens, or damaged cells. The mannose-binding lectin pathway can be activated by C3 hydrolysis or antigen without the presence of antibodies (non-specific immune response). In all three pathways, C3-Convertase cleaves and activates components C3, forming C3a and C3b, and further causing a cascade of cleavage and activation events. C3b binds to the surface of pathogens, leading to greater internalization by phagocytic cells by opsonization.

In the alternative route, C3B connects to Factor B. Factor D Releases Factor B from Factor B to C3B. The complex of C3b (2) Bb is a protease that connects C5 to C5b and C5a. C5 converts are also formed by the classical pathway when C5b is bound to C4b and C2b. C5a is an important chemotactic protein, which helps to recruit inflammatory cells. C3a is a precursor to an important cytokine called adipokine which is cell signaling protein (Klos, *et al.* 2013) [6] and is generally rapidly cleaved by carboxypeptidase B. In both C4a and C5a Anaphylatoxin activity occurs, which directly triggers mast degeneration and increasing contraction of cells as well as vascular and muscle permeability. C5b membrane attack pathways activates, resulting in the initiation of membrane attack complex (MAC), including C5b, C6, C7, C8, and the polymer C9 (Goldman and Prabhakar, 1996) [4]. MAC is the cytolytic end product of the complement cascade; it forms a transmembrane channel, which causes osmosis in lymphatic system. Kupffer cells and other macrophage cell types help clear complement-coated pathogens.

Materials and methods
Patient recruitment
During the year 2017 to 2019, medically diagnosed pneumonic patients were admitted from the Shyam Shah Medical College, Medicine Department (OPD) of Rewa (M.P.), 240 pneumonic patients were recruited for the current investigation.

All of the recruits were of central Indian origin, mostly from Rewa, Satna, Sidhi, Singrauli and Shahdol. Diagnosis of pneumonia was based on measurement of ESR (Erythrocyte Sedimentation Rate) on people suffering from pneumonia.

Healthy controls
240 randomly selected healthy control (HC) was enrolled in the study. The control group included Rewa, Satna, Sidhi, Singrauli, Shahdol, as well as medical staff and healthy

volunteers with persons living in the central region of India. Therefore, with the same environmental and social factors as the equal average age and gender ratio, the control group was created from the same area.

Sample collection strategy

About 5 ml Blood samples were collected in 0.5 M EDTA coated vials with healthy palm along with each pneumonia. Other information and clinical profile and matters and control topics was filled in a detailed proforma.

Quantitative measurement of Anaphylatoxin C5a

The BD CBA Human Anaphylatoxin Kit (Catalog No. 561418) is used to quantitatively measure anaphylatoxin C5a protein levels in a single EDTA plasma or serum sample (Loffler *et al.* 2010) [7].

Principle

BD CBA assays provides a way to capture a soluble analyte or set of analytes with beads of known size and fluorescence, making it possible to detect analytes using ELISA Kit Each capture bead in the kit has been conjugated with a specific antibody. The detection reagent provided in the kit is a mixture of phycoerythrin (PE) conjugated antibodies, which provides a fluorescent signal in proportion to the amount of bound analyte. When the capture beads and detector reagent are incubated with an unknown sample containing recognized analytes, sandwich complexes (capture bead + analyte + detection reagent) are formed. These complexes can be measured using ELISA to identify particles with fluorescence characteristics of both the bead and the detector. Three bead populations with distinct fluorescence intensities have been coated with capture antibodies specific for C5a plasma proteins and their desArg forms. In plasma and serum, C5a are rapidly converted to their desArg forms (C5a desArg). The Human Anaphylatoxin kit measures all C5a and their desArg forms (since this kit will measure both forms of each protein, normal and desArg, this manual will use C5a when referring to the measurement of either form). The three bead populations are mixed together to form the bead array.

During the assay procedure, you will assemble the anaphylatoxin capture beads with standards (purified from human plasma) or test samples (EDTA plasma or serum), incubate, wash and then incubate with the PE conjugated detection antibodies to form sandwich complexes. In this assay, the Human Anaphylatoxin Standards consist of purified C5a desArg.

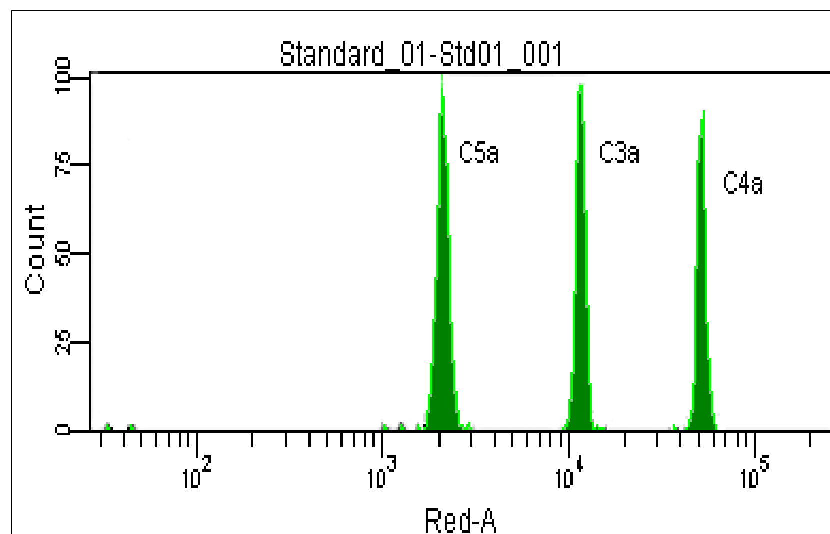

Fig 3: Quantitative measurement of Anaphylatoxin (C5a)

Bead reagents Human Anaphylatoxin Capture Beads (A1–A3): An 80- test vial of each specific capture bead (A1–A3). The specific capture beads, having discrete fluorescence intensity characteristics are distributed from brightest (A1) to dimmest (A3).

Setup Beads (D): A 30-test vial of setup beads for setting the initial instrument PMT voltages and compensation settings is sufficient for 10 instrument setup procedures. The ELISA Setup Beads are formulated for use at 50 µl per test.

Antibody and standard reagents

Human Anaphylatoxin PE Detection Reagent (B): An 80-test vial of PE-conjugated anti-human C5a antibodies, formulated for use at 50 µl per test. Human Anaphylatoxin Standards (C): Two vials containing lyophilized human proteins purified from serum. Each vial should be reconstituted in 2.0 ml of Assay Diluent to prepare the top standard. PE Positive Control Detector (E1): A 10-test vial of PE conjugated antibody control that is formulated for use at 50 µl per test. This reagent is used with the ELISA Setup Beads to set the initial instrument compensation settings. FITC Positive Control Detector (E2): A 10-test vial of FITC-conjugated antibody control that is formulated for use at 50 µl per test. This reagent is used with the ELISA Setup Beads to set the initial instrument compensation settings. Buffer reagents Wash Buffer (F): One 260-ml bottle of phosphate buffered saline (PBS) solution (1X), containing protein and detergent used for wash steps and to resuspend the washed beads for analysis. Assay Diluent (G): Two 30-ml bottles of a buffered protein solution (1X) used to reconstitute and dilute the Human Anaphylatoxin Standards and to dilute test samples.

Procedure To reconstitute and serially dilute the standards

1. Open a vial of lyophilized Human Anaphylatoxin Standards. Transfer the standard spheres to a 15-ml polypropylene tube. Label the tube “Top Standard.”

2. Reconstitute the standards with 2 ml of Assay Diluent.
a. Allow the reconstituted standard to equilibrate for at least 15 minutes at room temperature.
b. Gently mix the reconstituted protein by pipette only. Do not vortex or mix vigorously.

3. Label eight 12 × 75-mm tubes and arrange them in the following order: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256.
4. Pipette 300 µl of Assay Diluent in each of the 12 x 75-mm tubes.
5. Perform serial dilutions.

Assay preparation
a. Transfer 300 µl from the Top Standard to the 1:2 dilution tube and mix thoroughly by pipette only. Do not vortex.
b. Continue making serial dilutions by transferring 300 µl from the 1:2 tube to the 1:4 tube and so on to the 1:256 tube.
c. Prepare one 12 x 75-mm tube containing only Assay Diluent to serve as the 0 pg/ml negative control.

Mixing the beads to mix the Capture Beads:
1. Determine the number of assay tubes (including standards and controls) that are required for the experiment (eg, 8 unknowns, 9 anaphylatoxin standard dilutions and 1 negative control = 18 assay tubes).
2. Vigorously vortex each Capture Bead suspension for 3 to 5 seconds before mixing.
3. Add a 20-µl aliquot of each Capture Bead, for each assay tube to be analyzed, into a single tube labeled "Mixed Capture Beads" (for example, 20 µl of C4a Capture Beads × 18 assay tubes = 360 µl of C5a Capture Beads required).
4. Vortex the bead mixture thoroughly.
5. Dilute test samples by the desired dilution factor (for example, 1:200, 1:400 or higher) using the appropriate volume of Assay Diluent and Mix sample dilutions thoroughly.

To perform the assay
1. Vortex the mixed Capture Beads and add 50 µl to all assay tubes.
2. Add 50 µl of the Human Anaphylatoxin Standard dilutions to the control tubes as listed in the following table.

Table 1: The Human Anaphylatoxin Standard dilutions to the control tubes.

Tube Label	Concentration (pg/ml)	Anaphylatoxin Standard dilution
1	0 (negative control)	no standard dilution (Assay Diluent only)
2	4	1:256
3	8	1:128
4	16	1:64
5	32	1:32
6	62.5	1:16
7	125	1:8
8	250	1:4
9	500	1:2
10	1000	Top Standard

3. Incubate the assay tubes for 2 hours at room temperature, protected from light.
4. Add 1 ml of Wash Buffer to each assay tube and centrifuge at 200*g* for 5 minutes.
5. Aspirate and discard the supernatant from each assay tube carefully.
6. Add 50 µl of the Human Anaphylatoxin PE Detection Reagent to all assay tubes.
7. Incubate the assay tubes for 1 hour at room temperature, protected from light.
8. Add 1 ml of Wash Buffer to each assay tube and centrifuge at 200*g* for 5 minutes.
9. Carefully aspirate and discard the supernatant floating on the surface from each assay tube and leave it.
10. To resuspend the bead pellet Add 300 µl of wash Buffer to each assay tube.
11. Wet the plate by adding 100 µl of Wash Buffer to each well.
12. Place the plate on the vacuum manifold.
13. Aspirate for 2 to 10 seconds until the wells are drained.
14. Remove the plate from the manifold, then blot the bottom of the plate on paper towels.
15. Add 50 µl of each of the following to the wells in the filter plate:
a. Capture Beads (vortex before adding)
b. Standard or sample (add standards from the lowest concentration to the highest followed by samples)
16. Cover the plate and shake it for 5 minutes at 600 rpm on a plate shaker.
17. On a non-absorbent, dry surface, incubate the plate for 2 hours at room temperature.
18. Remove the cover from the plate and apply the plate to the vacuum manifold.
19. Aspirate vacuum for 2 to 10 seconds until the wells are drained.
20. Remove the plate from the manifold, then blot the bottom of the plate on paper towels after aspiration.
21. Add 200 µl of Wash Buffer to each well. Cover the plate and shake for 2 minutes at 600 rpm.
22. Repeat step 7 through step 9.
23. Add 50 µl of Human Anaphylatoxin PE Detection Reagent to each well.
24. Cover the plate and shake it for 5 minutes at 600 rpm on a plate shaker.
25. Incubate the plate for 1 hour at room temperature on a non-absorbent, dry surface.
26. Add 120 µl of wash buffer to each well to resuspend the beads.
27. Cover the plate and shake it for 2 minutes at 600 rpm before you begin sample acquisition.

Calculations
Calculated the mean absorbance for every set of reproduction standards, controls and samples, and subtract the average zero standard optical density. Plot the standard curve on log-log graph paper, with widespread attention on the x-axis and absorbance on the y-axis. Drawn the best-fit straight line through the standard points. For samples that have been diluted, the attention study from the general curve has to be expanded by using the dilution component to decide the genuine awareness of the goal protein existing (Naito, *et al.* 2006) [8].

Results

Clinical profile of patients and control

Clinical profile of patients and control table 2 indicates attributes on enrollment in age, residence and ethnicity of pneumonia and healthy control group. Within the given attribute, the variations between these 2 groups are equally and statistically non-significant, these are vital for keeping an equivalent 2 groups all told the norms apart from the study taken.

Table 2: To show the clinical characteristics of pneumonic patients and control in this study.

S.N.	Characteristic	Pneumonic Patients	Healthy control
1	No. of subjects	240	240
2	Male female ratio	88:152	98:142
3	Children: Adult	210:30	198:42
4	Mean Age (in year)	14.7	17.2
5	Age range (in year)	1-26	4-38
6	Mean weight (in Kg)	18.12	20.34

The number of patients and control for every cluster is 240 for study. The male feminine quantitative relation for case and control severally was 88:152 and 98:142. Children: Adult quantitative relation between groups 210:30 and 198:42 was for case and control. The average age of the case was 14.7 years and it had been adjusted to 17.2 for management. Average weight was 18.12 and 20.34 was for case and control, severally.

Association of C5a between pneumonic patients and control

C5a is a mediator of the inflammation process that acts as a chemotactic agent for neutrophils and increase high on the inflammation site. C5a is a splitting product of the compliment system and plays a vital role in the generation of inflammatory response against infections through hypersensitivity reactions. The increasing concentration of C5a in the blood is a symptom of pneumonic inflammatory reactions in the body. In order to analyze the role of C5a in pneumonic patients, the blood concentration of C5a of the case and control was measured and the differences in concentration were calculated statistically and the results are presented in the table 4.11. The observation of present study shows the significant elevation of the C5a blood concentration in the group of pneumonic patients.

The results of the C5a analysis indicated that C5a are found in normal blood level in healthy controls. Average value of blood C5a concentration was seen 23.5 ± 1.31 ng/ml in pneumonic patients and 5.7 ± 0.86 ng/ml in healthy controls. The median value of C5a blood concentration was found 90 pg/ml and 40 pg/ml. Standard error of the mean was found 8 pg/ml and 4 pg/ml respectively for patients and control. The value of t-test of the differences of C5a blood concentration between pneumonic patients and healthy control was found 173.59 with 998 degree of freedom which is statistically significant at the level of P <0.0001. Limit of C5a blood concentration in the patient was 3-28 ng/ml and 4-7 ng/ml was found in the control group.

Table 3: Comparison of the C5a concentration in blood of pneumonic patients to control using t-test (unpaired).

S.N.	Parameters	Pneumonic patients	Healthy controls	t-test P value
1.	Mean ± SD	23.5 ± 1.31 ng/ml	5.7 ± 0.86 ng/ml	P<0.0001 t=173.59 df=998
2.	Median pg/ml	90	40	
3.	SEM pg/ml	8	4	
4.	Range ng/ml	3-28	4-7	

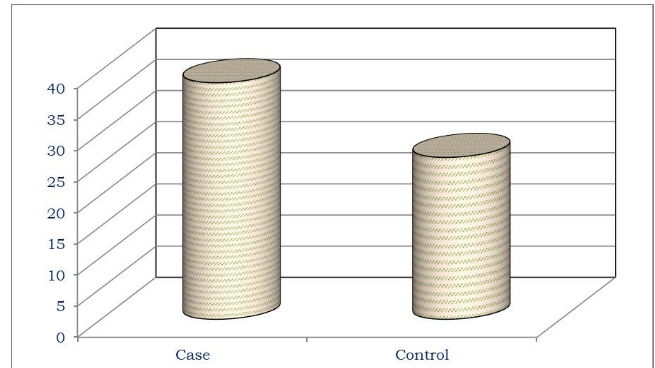

Fig 1: Comparison of C5a concentration in blood of pneumonic patients to control.

Discussion

Complement protein C5a has a very short half-life, which makes regular blood samples difficult to measure. Complement activation by pneumolysis was first found in pulmonary pneumococcal development and to promote pneumococcal species in mice (Sekowska *et al.* 2006) [10]. In a recent study victimisation enzyme-linked immunosorbant assay technique, it indicates that patients with acute lymphatic disease in C5a had an increase, which would include musculoskeletal and neurological symptoms developed within 96 hours of tick-bite (Kotton and Morrisey, 2014) [5].

This study found that the level of C5a was common in pneumonia patients. Although different studies in two studies can be due to various techniques used to measure C5a, our results were validated by the level of complement activation in patients of our pneumonic patients, which were previously mentioned in this autoimmune disease, Levels were the same. Thus, C5a seems to be a helpful marker for both clinical diagnosis and treatment of patients suffering from pneumonia (O'Garra *et al.* 2004) [9].

Conclusion

C5a is a mediator of the inflammation process and acts as a chemotactic agent for neutrophils and increase extravasations on the inflammation site. C5a is a splitting product of the compliment system and plays a vital role in the generation of inflammatory response against infections through anaphylactic reactions. The exaggeratedconcentration of C5a in the blood is a sign of pneumonic inflammatory reactions within the body.

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