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The analysis of Anaphylatoxin C3a with pneumonia patients in Rewa region

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Abstract
The present paper deals the analysis of C3a with Pneumonia patients in Rewa region. The results of C3a analysis indicated that C3a has a normal blood level in healthy controls. The average value of blood C3a concentration was seen 146 ± 4.04 ng/ml and 94 ± 2.51 ng/ml pneumonic patients and healthy controls respectively. The value of median was found 108 pg/ml and 64 pg/ml. Standard error of the mean was found 6 pg/ml and 3 pg/ml respectively for patients and control. The value of t-test of C3a blood concentration between pneumonic patients and healthy control was found 63.54 with the degree of freedom which is statistically significant at the level of P <0.0001. The median value of C3a blood concentration was 108 pg/ml and 64 pg/ml. The limit of the C3a blood concentration was found 78-220 pg/ml in the patients and 78-108 pg/ml in the control group.

Keywords: Pneumonia, C3a, Rewa region

Introduction
Pneumonia is a common respiratory organ infection characterized by assortment of pus and fluids within the lungs, alveoli (air sacs). Alveoli are structures that facilitate the exchange of gases and assortment of pus in them makes respiration tough. 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]. To know this condition the pathophysiology of pneumonia needs to be understood. Whenever someone suffers from respiratory organ injury because of chemical irritants or physical factors the condition is also stated as pneuminitis and this is differentiated from the condition of infectious pneumonia. In understanding the pathophysiology of pneumonia, it could be understand that this condition affects people of all ages. The incidence of respiratory disease pneumonia is however higher among younger kids and older people beside those people has a compromised immune system (Black, 2003) [2].

Pneumonia is typically caused by invasion of pathogens in human host. In adults it's largely caused by bacterium, whereas in kids and infants it is usually because of viruses. Physical or chemical injury to the lungs can also lead to this condition. People of United Nations are hospitalized and have semi-permanent sickness like bronchial asthma, heart condition, cancer, HIV/AIDS, respiratory organ diseases or polygenic diseases are at a better risk of developing Hospital-acquired pneumonia is additionally common.

The Central Bureau of Health Intelligence of the MoHFW reported ARI (Acute Respiratory Infections) mortality ranging from 3200 to 6900 each year, giving a mortality rate of 0.32% to 0.61% deaths per l,00,000 population. The WHO and UNICEF estimated that approximately 4,08,000 under-five deaths in India are contributed by pneumonia (Rudan *et al.* 2008) [3]. A large modelling analysis of data from 193 countries calculated that pneumonia contributes 18% of a total of 8.795 million under-five deaths (Black *et al.* 2008) [4].

Many cytokines found in the extracellular fluid interact with pulmonary infections (different effects on different types of target cells) at any time during an immunological response. These cytokines are nonspecific (many cytokines have the same effect), synergistic (many Cooperative effect of cytokines), antagonistic (inhibiting one cytokines effect by another) and cascade inductive (inhibition of a particular cytokine) Multiple-step feed-ahead mechanism for increased production etiquette.

These interactions make it possible for coordinated recruitment and activation of an entire network of immune cells by the relatively small number of cytokines produced by a single cell type (such as macrophages or Th cells).

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 and 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 C3a

The BD CBA Human Anaphylatoxin Kit (Catalog No. 561418) is used to quantitatively measure anaphylatoxin C3a protein levels in a single EDTA plasma or serum sample (Loffler *et al.* 2010) [5].

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 C3a plasma proteins and their desArg forms. In plasma and serum, C3a are rapidly converted to their desArg forms (C3a desArg).

The Human Anaphylatoxin kit measures all C3a and their desArg forms (since this kit will measure both forms of each protein, normal and desArg, this manual will use C3a 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 C3a desArg.

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 C3a Capture Beads × 18 assay tubes = 360 µl of C3a 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.
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) (2) 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.

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	10	1:256
3	20	1:128
4	40	1:64
5	80	1:32
6	156	1:16
7	312	1:8
8	625	1:4
9	1250	1:2
10	2500	Top Standard

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) [6].

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 C3a between pneumonic patients and control

To analyze the role of C3a in the susceptibility marker in pneumonic patients, the blood concentration of C3a of the case and control was measured and the difference in concentration was calculated statistically and the results are presented in table 3. Observations of the current study show a significant increased level of C3a blood concentration in the group of pneumonic patients.

The results of C3a analysis indicated that C3a has a normal blood level in healthy controls. The average value of blood C3a concentration was seen 146 ± 4.04 ng/ml and 94 ± 2.51 ng/ml pneumonic patients and healthy controls respectively. The value of median was found 108 pg/ml and 64 pg/ml. Standard error of the mean was found 6 pg/ml and 3 pg/ml respectively for patients and control. The value of t-test of C3a blood concentration between pneumonic patients and healthy control was found 63.54 with the degree of freedom which is statistically significant at the level of P <0.0001. The median value of C3a blood concentration was 108 pg/ml and 64 pg/ml. The limit of the C3a blood concentration was found 78-220 pg/ml in the patients and 78-108 pg/ml in the control group.

Table 3: Comparison of the C3a concentration in blood of pneumonic patients to control by using t-test (unpaired).

S.N.	Parameters	Pneumonic patients	Healthy controls	t-test P value
1.	Mean ± SD	146 ±4.04 ng/ml	94±2.51 ng/ml	P<0.0001 t=63.54 df=998
2.	Median pg/ml	108	64	
3.	SEM pg/ml	6	3	
4.	Range pg/ml	78-220	78-108	

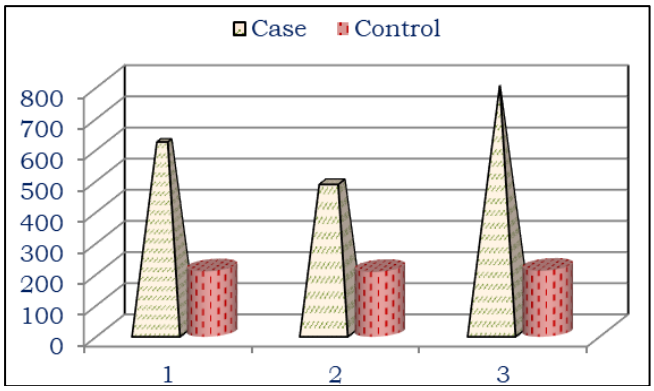

Fig 2: Comparison of C3a concentration in blood of pneumonic patients to control.

Discussion

C3a is a splitting product of the compliment system and plays a very important role in the generation of inflammatory response against infections through hypersensitivity reactions. C3a is a mediator of the inflammation process that acts as a chemotactic agent for neutrophils and will increase further on the inflammation site. The enlarged concentration of C3a in the blood indicates pneumonic inflammatory reactions in the body.

In addition, C3a receptor-deficient mice manufacture additional IL-1 once administration of blood vessel lipopolysaccharide compared to wild-type mice (Black *et al.* 2008) [4].

This study found that the level of C3A common in pneumoniae patients. Although different studies can be due to various techniques used to measure C3a, our results were valid for the level of supplemental activation in patients of our pneumonia, which were previously mentioned in this autoimmune disease. Levels were the same (O'Garra *et al.* 2004) [7].

Conclusion

C3a is a splitting product of compliment system which plays a vital role in the generation of inflammatory response against infections through anaphylactic reactions. C3a is a mediator of the inflammation process which acts as a chemotactic agent for neutrophils and increases additional compliment proteins on the inflammation site. The increased concentration of C3a in the blood indicates pneumonic inflammatory reactions in the body.

To analyze the role of C3a as a susceptibility marker in the pneumonic patients, the blood concentration of the C3a of the case and healthy control was measured and also the difference in concentration was calculated statistically. The observation of this study shows a significant elevation of C3a blood concentration in the cluster of pneumonic patients.

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