A Rapid Point of Care CC16 Kit for Screening of Occupational Silica Dust Exposed Workers for Early Detection of Silicosis/Silico-Tuberculosis

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Abstract

Silicosis is an irreversible, incurable and progressive occupational disease caused by prolonged exposure to crystalline-silica while working in related industries. Conventionally diagnosis is done by chest radiology in advanced stage as early symptoms often go unnoticed. Early detection and secondary prevention could be the only realistic possible control strategy for controlling silicosis as no other method of treatment is available. Additionally, these patients are also vulnerable to drug resistant tuberculosis. Developing countries like India has a huge burden of silico-tuberculosis. Hence, a rapid and inexpensive screening method is a need for early detection of silicosis among silica dust exposed workers. Serum CC16 is a useful proxy screening marker for early detection of silicosis. In this study a lateral-flow assay for semi-quantitative estimation of serum CC16 level was developed. The detection was performed using gold nanoparticles conjugated anti-CC16 monoclonal antibodies. A sum of 47 serum samples was tested to do performance evaluation of the assay. A concentration of 6ng/ml or less produced one band, 6.1 – 9 ng/ml produced two bands, while more than 9 ng/ml produced all the three bands at the test zone. This assay can be used as a proxy marker for periodic screening and early detection of silicosis in vulnerable workers.

Introduction

Club cell protein 16 (CC16) is the most abundant protein in broncho-alveolar secretions. CC16 inherits an anti-inflammatory property in lungs. Chronic Obstructive Pulmonary disease (COPD) is caused due to exposure of lungs to smoke or any other particulate pollutants[1]. CC16 has a molecular mass of 16 kilo Dalton, and belongs to the secretory globin sub-family of proteins. CC16 is a homodimeric protein with identical 70-amino acid subunits linked in an antiparallel orientation by two disulfide bonds[2].

The main source of CC16 is the Club cell (formerly called Clara cell) which is a non-ciliated, non-mucous secreting club-shaped cell present mainly in bronchioles as well as basal cells found in large airways. The density of Club cells throughout the respiratory tract varies substantially between species. In humans, Club cells represent 1% of all airway epithelial cells in the bronchioles and 5% in the respiratory bronchioles[3]. Other organs that contain CC16-secreting cells are the prostate, ovaries, pancreas, mammary glands, and uterine endometrium[4, 5].

As per the literature data, many chronic pulmonary inflammatory diseases such as anthraco-silicosis, chronic obstructive pulmonary disease (COPD), asthma etc. causes depletion of CC16. COPD is a condition of lungs in which the pathogenesis is still not very clear. The main reason being progressive symmetric erythro-keratodermia develops during COPD causing hindrance in the airway. The etiological factors can be many such as breathing in of particulate dust particles, bacterial infections or smoking. In all types of cases, Club cells are degenerated and reduced in number resulting in decreased levels of CC16 in Broncho-alveolar lavage fluid (BALF) and serum. The anti-inflammatory and protective effect of CC16 on the airway epithelium is gone resulting in inflammation of lungs. In case of per-acute or acute attacks, the CC16 level increases and results in the repair of the airway epithelium. But in cases of chronic

exposures, the CC16 levels reduce gradually resulting in the chronic inflammation which is ultimately leading towards fibrosis of lungs.

Silicosis is an irreversible occupational ailment of the respiratory system caused by the invasion of lung tissue (parenchyma) due to dust consisting of crystalline silica or silicon dioxide of respirable size (less than 10μ in diameter). Individuals with various exposure interval ranging from 2 to 15 years or more in the industries like mines, stone quarry, agate, construction sites and non-metallic product manufacturing units for example refractory (articles with heat resistant ability), ceramic, glass, mica, and structural clay are more prone to silicosis[6]. These micro-particles get trapped in the interstitial lung collagen tissue resulting in fibrosis of the lung. Therefore, it becomes one of the major occupational health hazards for the silica dust exposed workers working in relevant industries. Regrettably, most of the silicosis cases remain undiagnosed or misdiagnosed at an early stage due to asymptomatic or mild symptomatic nature of the initial stage of the disease, lack of suitable biomarker for early detection, poor health-seeking behavior of the workers and poor occupational health care delivery service at the working areas, particularly in unorganized sectors[7, 8, 9].

Patients with silicosis are prone to develop pulmonary tuberculosis also called silico-tuberculosis, probably due to destruction of alveolar macrophages. Differential diagnosis is difficult unless the physician is aware of the occupational history of silica exposure, which is very subjective in nature. Also, it becomes very difficult to differentiate between silicotic nodules and tuberculous infiltration in radiography. Additionally, the difficulty in isolation of *Mycobacterium tuberculosis* from sputum of silico-tuberculosis patients as silicotic fibrosis prevents discharge of *Mycobacterium* in the sputum, making the situation more difficult[10]. Hence, a suitable biomarker is required for early detection of silicosis. This will augment in the prophylaxis and control of advanced silicosis and silico-tuberculosis patients.

The diagnosis of silicosis needs to be confirmed by chest radiology following clinical examination with a history of occupational exposure to silica dust for a varying period. X-ray of chest shows bi-lateral pathognomonic nodular opacities in silicosis. Diagnosis is invariably made at an advanced or end stage when it is irreversible. Moreover, silicosis patients are susceptible to tuberculosis, which is often difficult to diagnose and treat resulting in drug resistant tuberculosis. Considering above, a suitable biomarker for early detection of silicosis is needed to protect these vulnerable workers.

A number of anti-inflammatory biomarkers for early diagnosis of silicosis have been attempted, but most of these were found to be non-specific and hence conferred unsuitable for diagnosis of lung related pathologies[11, 12]. Club cell protein (CC16) is secreted by Club cells of Broncho-alveolar epithelial tissue of the lung[13] (Bernard et al., 1994). CC16 is proposed to be a peripheral marker of respiratory epithelial injury that protects the respiratory tract against oxidative stress-induced inflammation[14, 15, 16] and passively diffuses in bronchoalveolar-blood barrier to plasma[17]. The serum concentration of CC16 can be used to decipher the degree of respiratory tract injury and lung alveolar capillary barrier integrity at an early stage. Though the exact physiological mechanism of CC16 remains unknown, but evidences suggest significant reduction of CC16 in silica dust-exposed workers with no change in respiratory symptoms, normal chest

radiology and lung function tests indicates that serum CC16 could be an early asymptomatic detection tool for silicosis among silica-exposed population at risk.

At present-day, the CC16 detection is performed with commercially available enzyme linked immunosorbent (ELISA) assays of clinical field application[18] (Biovendor – Laboratorni) that requires to be imported from the foreign countries. The available commercial assays are very expensive and the cost cannot be afforded by the daily wage workers. Thus, there is a need for economical, user friendly and rapid detection devices and methods which does not require expensive instrumentation or specialized skills for testing and analysis. Considering the huge burden of silicosis patients in the country, all primary health care centers need to have some kind of users' friendly point of care screening/diagnostic tool for early detection of silicosis so that occupational health care delivery services could be extended there. This will revolutionize the occupational health scenario of the country to a great extent focusing silicosis and silico-tuberculosis.

In the current study we describe a Point of Care assay that can be particularly employed for semi-quantitative estimation of CC16 in human serum samples. This assay can be used periodically at regular intervals to assess the serum CC16 levels among workers with history of silica dust exposure. This assay would give an idea about an estimated lung injury caused by silica dust exposure before advising for their radiological confirmation to arrive at a conformed diagnosis. So, it may be considered as a proxy bio-marker and screening tool for silicosis.

Result

The principle of this assay is the serum CC16 protein migrates by capillary action through the nitrocellulose membrane and reaches the test lines. At the test line a certain amount of serum CC16 is captured by the rabbit anti CC16 polyclonal antibodies. The excess amount of CC16 protein is trapped by the second test line followed by the third test line. This is followed by running GNPs conjugated with anti-CC16 monoclonal antibodies (anti CC16 mAb + GNPs complex) through the NCM which will produce a red colored band at the test line and the control line. The intensity and the number of red line developed at the CRP test zone were directly proportional to the concentration of CC16 in the sample semi quantitatively. This in turn can be used to predict the suspected degree of silicosis (lung damage) in the patients.

Interpretation of the results was done be checking the control line. The control line was indicative of valid reaction. If the control line does not appear, the reaction is invalid. If the serum CC16 concentration is 6 ng/ml or less, one red colored band was detected. If the serum CC16 concentration was in the range of 6.1 to 9 ng/ml then the assay produced two bands and if the serum CC16 concentration was more than 9 ng/ml, the assay produced three bands at the test zone. One control band is observed at the control line irrespective of the CC16 concentration present in the serum (Figure 1). Clinical significance of the results obtained in terms of the number of bands visualized and their relation with CC16 concentration is depicted in the table 1 and figure 1 below.

Performance evaluation of the assay

The performance of the assay was evaluated by testing 47 serum samples. The serum samples were also tested by commercially available ELISA kit. The comparative evaluation was performed by estimating CC16 concentration by ELISA and numbers of bands on lateral flow assay (Table 2). The comparative evaluation of ELISA and lateral flow strip test showed that out of 47 serum samples 6 samples were in the range of 0 to 6 ng/ml CC16 concentration. These six samples exhibited 1 band at the test zone and one band at the control line. In the range of 6.1 to 9 ng/ml concentration of CC16, there were 12 samples. These samples exhibited two bands at the test zone and one band at the control line. In the range of 9.1 and above concentration of CC16 there were 29 samples. These samples exhibited 3 bands at the test zone and one band at the control line. A gray zone was observed at the samples having a CC16 concentration of 9 to 9.5 ng/ml. There were four number of samples in that range of CC16 concentrations. Out of four, two samples exhibited two bands at the test zone and two samples exhibited three bands at the test zone. The details are mentioned in the table 3. The results of performance evaluation of semi-quantitative lateral flow assay with ELISA using 47 serum samples have been summarized in table 4.

Discussion

India has a huge burden of silicosis as it is conventionally diagnosed by chest X-ray, which often occurs in late stage due to the fact that initially it is with no or mild symptoms. Since there is no specific line of treatment, early detection and secondary prevention of silicosis is the only realistic solution for control of silicosis. There was no mechanism for early detection of silicosis till date as patients often report late for evaluation for silicosis. On the other hand, pathological process takes some time (few months to year/s) for development of typical opacities to be detected by the chest X-ray from the onset of silica dust exposure that leads to tissue inflammation and fibrosis. Considering above, early detection of silicosis is an immediate need of the day. It is also needed for elimination of tuberculosis as silicosis patients are vulnerable to tuberculosis & often become drug resistant tuberculosis due to declined lung immunity and anti-tubercular drugs do not reach to infected tissue in sufficient quantities due to fibrosis. India has a huge burden of silicosis and most workers are from informal economy sectors without much socioeconomic & medical support. Under the above situation, serum CC16 kit should be used as a tool for early detection of silicosis through periodic screening among silica dust exposed workers. The study conducted by ICMR-National Institute of Occupational Health, Ahmedabad, had conclusively evidenced that serum CC16 is a useful proxy marker for early detection of silicosis among silica dust exposed workers. Subsequently another study showed that when serum CC16 value ranges between 6.1 and 9 ng/ml, it is indicative of early silicosis, which needs to be confirmed by chest X-ray or CT scan for further necessary action including notification, health management and compensation. Hence, a semi-quantitative kit has been developed by ICMR that would detect the suspected early silicosis among silica dust exposed workers. This kit is intended for periodic screening among silica dust exposed workers for early diagnosis & prevention/control of silicosis and to be managed by the primary health care workers in

the entire country. Necessary logistical supports & training of primary health workers are essential components towards these initiatives.

The principle of this assay is the serum CC16 protein migrates by capillary action through the nitrocellulose membrane and reaches the test lines. At the test line a certain amount of serum CC16 is captured by the rabbit anti CC16 polyclonal antibodies. The excess amount of CC16 protein is trapped by the second test line and the same is followed by the third test line. This is followed by running GNPs conjugated with anti-CC16 monoclonal antibodies (anti CC16 mAb + GNPs complex) through the NCM which will produce a red colored band at the test line and the control line. The intensity and the number of red line developed at the CRP test zone were directly proportional to the concentration of CC16 in the sample semi quantitatively. This in turn can be used to predict the suspected degree of silicosis (lung damage) in the patients.

Interpretation of the results was done be checking the control line. The control line is indicative of valid reaction. If the control line does not appear, the reaction is invalid. If the serum CC16 concentration is 6 ng/ml or less, one red colored band is detected. If the serum CC16 concentration is in the range of 6.1 to 9 ng/ml then the assay produces two bands and if the serum CC16 concentration is more than 9 ng/ml, the assay produces three bands. One control band is observed at the control line irrespective of the CC16 concentration present in the serum.

The performance of the assay was evaluated by testing 47 serum samples. The serum samples were also tested by commercially available ELISA kit. The comparative evaluation was performed by estimating CC16 concentration by ELISA and numbers of bands on lateral flow assay. The comparative evaluation of ELISA and lateral flow strip test showed that out of 47 serum samples 6 samples were in the range of 0 to 6 ng/ml CC16 concentration. These six samples exhibited 1 band at the test zone and one band at the control line. The patients having 0 to 6 ng/ml of CC16 concentration are suspected to have either moderate (3.1 to 6 ng/ml) or advanced (0 to 3 ng/ml) silicotic lung injury. In the range of 6.1 to 9 ng/ml concentration of CC16, there were 12 samples. These samples exhibited two bands at the test zone and one band at the control line. The patients having serum CC16 value of 6.1 to 9 ng/ml are suspected to have a mild or early silicosis that is detectable by chest x-ray/CT scan for confirmation. Since smoking usually reduces mean serum CC16 value by 1 to 2 ng/ml compared to non-smokers, 6.1 to 9 ng/ml will include early silicosis both in non-smoking as well as smoking workers, considering a cut off value of 9 ng/ml or less for non-smoking and 1 to 2 ng/ml less in smokers. Hence, the range is made 6.1 to 9 ng/ml clubbing both categories together for operational ease.

In the range of 9.1 and above, concentration of CC16 there were 29 samples. These samples exhibited 3 bands at the test zone and one band at the control line. The patients having serum value of more than 9 ng/ml of CC16 but up to 12 ng/ml may be in their very early pathological process of silicosis which will not cast shadow by chest x-ray. Value above 12 ng/ml are usually healthy individuals. The present study has considered two issues for diagnosis of silicosis at an early stage – serum CC16 value by screening and chest x-ray/CT scan for confirmation. Considering above, the range of 6.1 to 9 ng/ml appears to

fulfill both and hence has been chosen for early detection range for various practical purposes (health management, notification & compensation etc.). Also gray zone was observed at the samples having a CC16 concentration of 9 to 9.5 ng/ml. There were four number of samples in that range of CC16 concentrations. Out of four, two samples exhibited two bands at the test zone and two samples exhibited three bands at the test zone. It is understood that for any biological event, there is some variation of values at an end. Hence, above event is expected as very few cases could be outside the designated range. There is not much other variation of serum CC16 with other demographic factors such as age sex etc. as was observed in earlier study conducted by ICMR-NIOH.

In conclusion, we describe a Point of Care assay that can be particularly employed for semi-quantitative estimation of CC16 in human serum samples. This assay can be used to assess the serum CC16 levels of the workers with silica dust exposure history for early detection of silicosis for various purposes such as notification, management and compensation as per law/guidelines of the country.

Methods

Raw materials

In the present study, the lateral flow membranes were purchased from MDI Membrane technologies Ambala, India. There are many types of membranes available out of them CNPF-SN12-L2-P25 10μm membrane was used in this study. The CNPF-SN12 membrane is associated with lower protein binding. The L2 means Laminate with NC membrane mounted on it and adhesive placed for sample pad and absorbent pad. The 10μm is the porosity of the nitrocellulose (NC) membrane.

The antibodies used in this study were procured from commercial sources. The antibody used for coating the membrane was rabbit anti human CC16 polyclonal antibody and it was purchased from (Catalogue No. 500-P330) PeproTech, USA. The antibody used for conjugation with gold nanoparticles (GNPs) was anti-CC16 monoclonal antibody and it was purchased from (Catalogue No. MA1-40223) Thermo Fisher scientific, USA.

The recombinant protein produced in *E. coli* was used as the standard in this assay. The recombinant club cell protein was a 9.2 kDa size consisting of 80 amino acids. This was purchased from (Catalogue No. RD191022200) Biovendor – Laboratorni medicina a.s. Czech Republic.

The colloidal spherical gold nanoparticles were purchased from (Catalogue No. 741981-25ML) Sigma Aldrich USA.

Samples collection

Ethical approval was obtained before initiating this study. A total of samples were collected by ICMR-NIOH from occupational health clinics in Jodhpur and Delhi and were transported to ICMR-NIV, Mumbai unit, for the purpose of this study after completion of NIOH's initial research work including confirmation silicosis by chest x-ray. About 3 ml blood sample was collected by venipuncture using a vacutainer from

each eligible and consented participant. The blood samples were allowed to coagulate by keeping the collection tubes in a slant manner. After coagulation of blood, the tubes were centrifuged at 1500 rpm for 5 minutes to separate the serum. The serum was separated in cryo-vials and stored at -20^{o}C till further use.

ELISA for the quantitation of CC16

The CC16 concentrations in serum samples were determined using a commercially available ELISA kit (Catalogue No. RD191022200 Biovendor – Laboratorni medicina a.s. Czech Republic). This is an antigen capture type ELISA which is also known as antigen sandwich ELISA. The ELISA was performed as per instruction manual provided by the kit and a brief description of the protocol is as follows.

The kit consists of a pre-coated ELISA strip in which anti-CC16 polyclonal antibody is coated. The serum samples as well as the standard protein samples were diluted 25 times (5 µl serum in 120 µl dilution buffer) and allowed to react with the pre-coated ELISA strips for 60 minutes at room temperature with shaking at 300 rpm followed by a quick wash. Then the biotin labelled polyclonal anti-human club cell protein antibody was added and incubated with captured club cell protein for 60 minutes. After another washing, streptavidin-horseradish peroxidase conjugate was added. After 60-minute incubation and the last washing step, the remaining conjugate was allowed to react with the substrate solution (TMB). The reaction was stopped by addition of acidic solution and the absorbance of the resulting yellow product was measured using TECAN M200 ELISA plate reader.

Conjugation of antibody with Gold Nanoparticles (GNPs)

A volume of 2 ml of mono dispersed GNPs solution (40 nm, negative charge) was taken in two separate sterile 1.5 ml tubes (1 ml in each tube). GNPs solution was centrifuged at 13200 rpm at 4^{o}C for 5 min. 500µl of supernatant was discarded from each tube and the soft pellets were resuspended in the remaining solutions.

Contents of the two tubes were pooled together. The pH of the pooled GNPs solution was adjusted to 9 using 0.1M K_2CO_3 solution. Generally, 12-15 µl of 0.1 M K_2CO_3 solution is enough to adjust the pH to 9 for 1 ml GNPs solution. 10 µg of anti-CC16 mAb in 10mM Tris HCl (pH 8) was added to the above prepared 1 ml GNPs solution drop wise with gentle mixing by inverting 5 times.

The tube was incubated at room temperature for 10 minutes. Bovine serum albumin (BSA) at a final concentration of 0.025% (W/V) was dissolved in 10mM Tris HCl (pH 8). Said buffer was added drop wise for blocking unoccupied sites on the GNPs mixed by inverting the tube 5 times. The tube was incubated at room temperature for 10 minutes. The reaction mixture was centrifuged at 4000Xg for 1 hour at 4^{o}C.

Maximum possible supernatant was discarded carefully without disturbing the soft pellet. The soft pellet was resuspended in 50 µl of 10mM Tris-HCl pH 8 with 0.1% (w/v) BSA. The conjugate containing tube was covered with aluminum foil and stored between 2 – 8^{o} C until further use. The working solution of

the conjugate was diluted 1:5 times (1 µl conjugate + 4 µl of 10mM Tris-HCl pH 8 with 0.1% (w/v) BSA buffer containing tween-20).

Lateral flow assay (LFA)

The CNPF-SN12-L2-P25 10µm membrane strips were used for this assay. Each strip having 0.5 cm width contains a 2.5 cm patch of nitrocellulose membrane (NCM) for the reaction.

The lateral flow strip was coated with three test lines and one control line on the NCM strip. The control line was located at the top (0.4 cm from the edge of NCM) and below that, there were three test lines located 0.4 cm from each other (Fig. 2). The control line was coated with the goat anti-mouse antibody 0.2 µg/µl in 10 mM Tris HCl buffer pH 8. The three test lines were coated with a specific capture antibody (Rabbit anti CC16 polyclonal antibody) in 10 mM Tris HCl buffer pH 8. After coating, the strips were allowed to dry at 37^{o}C for 1 hour. After drying the absorption pad was attached at the far end of the test strip in the direction of flow 1 mm overlapping the NCM.

Sample Application and Testing

The strips were placed at a horizontal surface.10 µl of each serum sample was loaded on each strip and allowed to flow through the membrane. 10 µl of working GNPs + anti-CC16 mAb conjugate was loaded and allowed to flow through the membrane strip. Again 10 µl of the wash buffer was allowed to flow through the membrane to wash off the extra GNPs + anti-CC16 mAb conjugate from the strip. Then the number of bands were observed on the lateral flow strip.

Performance evaluation of lateral flow assay

The performance of the assay was evaluated by testing 47 serum samples. The serum samples were also tested by a commercially available ELISA kit. The comparative evaluation was performed by estimating CC16 concentration by ELISA and numbers of bands on lateral flow assay. The statistical analysis was performed using standard online software.

Declarations

Ethics Statements:

In this study, the ethical approval was obtained from Ethics committee of Indian Council of Medical Research – National Institute of Occupational Health (ICMR-NIOH), Ahmedabad. A written informed consent was taken from all eligible participants for participating in this study voluntarily. All methods were carried out in accordance with relevant guidelines and regulations for the study.

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Author contributions:

S.S.N. conceived the idea, planned the experiments, supervised the study, analyzed the data, wrote and reviewed the manuscript. U.P.L. planned the experiments and performing the laboratory investigations, analyzed the data, wrote and reviewed the manuscript and S.A.S and T.G. were involved in performing the laboratory investigations and reviewed the manuscript. K.S. supervised the study, analyzed the data, and reviewed the manuscript. J.D. planned the experiments, supervised the study, analyzed the data and reviewed the manuscript. All the authors approved the manuscript.

Additional information

None

Competing interests

The authors declare no competing interests.

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Tables

Table 1: Depiction of the clinical significance with concentration of CC16 in serum.

Sr. No.	CC16 concentration (ng/ml)	Number of bands in lateral flow assay	clinical significance
1	0 to 6 ng/ml	1	Suspected moderate to advance silicosis
2	6.1 to 9 ng/ml	2	Suspected early silicosis
3	More than 9 ng/ml	3	Healthy or early silicosis not detectable by chest x ray

Table 2: Details of samples tested by ELISA and its comparison with lateral flow assay with the classification according to three categories of detection.

Sr. No.	CC16 concentration range by ELISA	No. of samples	Number of bands at test line	Matching	Un-matching
1	6 ng/ml or less	6	1	6	0
2	6.1 to 9 ng/ml	12	2	12	0
3	More 9.1 ng/ml	29	3	29	2

Table 3: Details of discordant samples and their comparison by ELISA and lateral flow assay.

Sr. No.	CC16 concentration by ELISA	Number of bands on test line	Match with ELISA results
1	9.5	2	No
2	9.5	2	No
3	9.5	3	Yes
4	9.545455	3	Yes

Table 4: Performance evaluation of lateral flow assay for semi-quantification of CC16 in 47 serum samples in comparison with ELISA.

CC16 concentration ng/ml	Nos. Samples	Nos. of bands on the strip			
		NIL	ONE	TWO	THREE
<=1	1	1	0	0	0
1.1 to 2	0	0	0	0	0
2.1 to 3	0	0	0	0	0
3.1 to 4	0	0	0	0	0
4.1 to 5	3	0	2	1	0
5.1 to 6	2	0	2	0	0
6.1 to 7	2	0	0	2	0
7.1 to 8	5	0	0	5	0
8.1 to 9	5	0	0	5	0
9.1 to 10	4	0	0	2	2
10.1 TO 11	6	0	0	0	6
11.1 TO 12	2	0	0	0	2
12.1 TO 14	3	0	0	0	3
14.1 TO 16	7	0	0	0	7
>16	7	0	0	0	7
Total	47		5	15	27

Figures

Sr. No.	Figure	Description	Sr. No.	Figure	Description
1		**Negative control** **Observation**: **Control line**: One band observed **Test line**: No band observed	2		**CC16: 0 to 6 ng/ml (Suspected moderate to advance silicosis)** **Observation**: **Control line**: One band observed **Test line**: Single band observed
3		**CC16: 6.1 to 9 ng/ml (Suspected early Silicosis)** **Observation**: **Control line**: One band observed **Test line**: Two bands observed	4		**CC16: > 9 ng/ml and above (healthy person or early silicosis, not detectable by X-ray)** **Observation**: **Control line**: One band observed **Test line**: Three bands observed

Figure 1

Detailed description for interpretation of results for semi-quantitative lateral flow assay for detection of CC16.

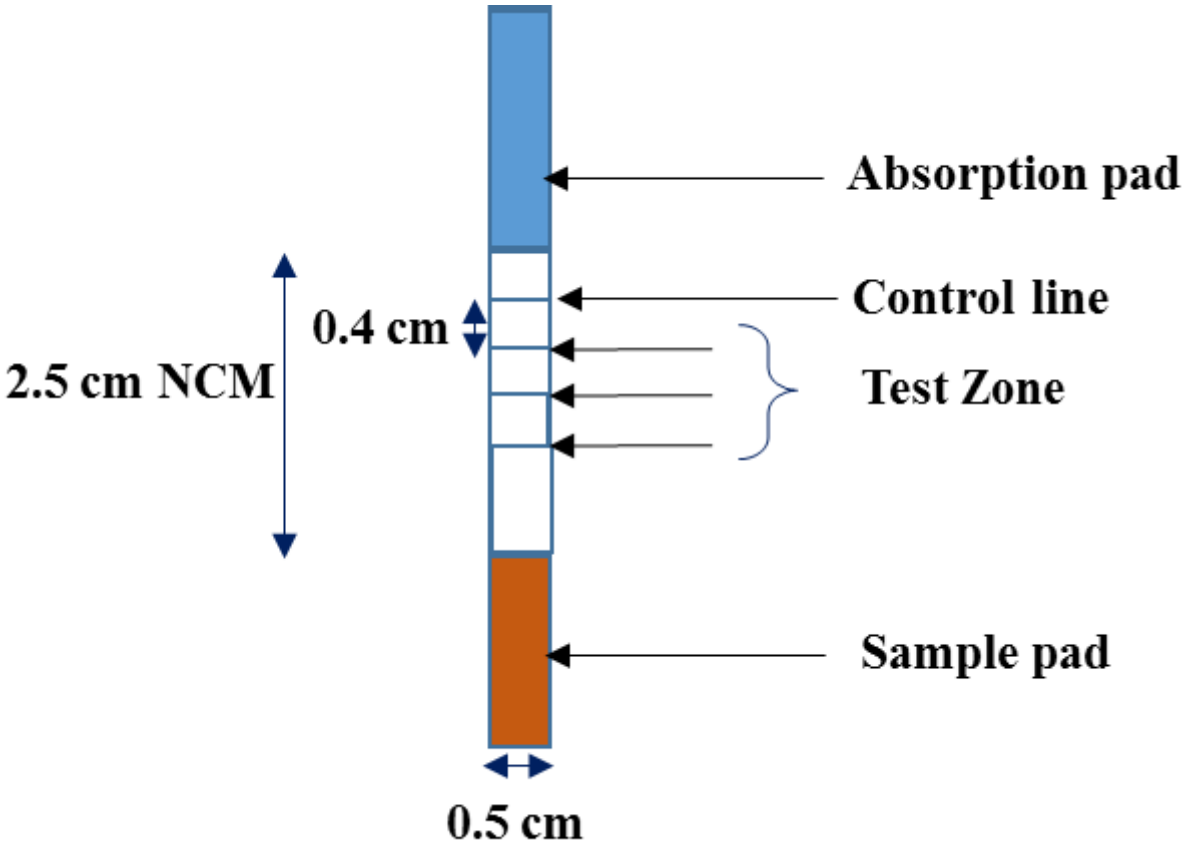

Figure 2

Schematic representation of lateral flow assay strip preparation. The figure describes a single strip of LFA with the width of 0.5 cm; length of NCM 2.5 cm and location of control line at the top and three test lines 0.4 cm below subsequently.