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SPOTTING BIOMARKERS OF PULMONARY TUBERCULOSIS IN HUMAN EXHALED BREATH USING PORPHYRIN BASED SENSOR ARRAY

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Abstract: In search of a rapid point-of-care diagnostic tool for the diagnosis of pulmonary tuberculosis (P-TB) this exhaled breath analysis study was conducted using porphyrin based sensor. A single total exhaled breath test was done on 62 participants, 32 P-TB cases and 30 healthy controls using sensor plate comprising of 24 coded porphyrin elements; Red, Green and Blue (RGB) analysis was done pre and post exposure. Different porphyrin derivatives exhibited varied RGB responses. Seven analytes in our sensor array showed notifiable responses to differentiate diseased from healthy in accuracy parameters: sensitivity was finest in three IB, IIBa, ICh, then IAb and ICd; in specificity ICa was best; Fair positive predictive value was in ICa, IIBa, ICd, I, IAb, ICh; likelihood ratio was not encouraging. ICa, IIBa, IB, IAb demonstrated fairly strong post-exposure chromatic changes in binary logistic regression. In our study, porphyrin array-based chemical sensing showed promising results for the direct diagnosis of pulmonary tuberculosis. The quality of interaction with organic volatile compounds can be controlled by suitable changes in the porphyrin macrocycle like peripheral substituent and central metal. This would lead to fine tuning of senor array by identifying responsive porphyrin derivatives with better predictive value and incorporation them in a single array for enhanced responses. It would be worth evaluating colorimetric porphyrin elements based sensor array in more detail as a likely cost-effective futuristic model of non-invasive entrant in diagnostic algorithms to halt the pandemic of tuberculosis.

Keywords: Porphyrin, Pulmonary Tuberculosis, Total Exhaled Breath, RGB analysis

INTRODUCTION

The rapid and accurate diagnosis of active tuberculosis remains a challenge till date. The mycobacterial infection inside the internal milieu of living cell produces volatile organic compounds typical to tuberculosis and comes out in breathe and can be used as a predictive parameter for case detection. The end products of mycobacterial metabolism in human breaths have the potential for the early markers in diagnosis, intervention as well as prognosis. In Mycobacterium tuberculosis cultures 130 different volatile organic compounds have been consistently detected (derivatives of benzene, naphthalene, and alkanes).[1] Several methods like Spectroscopy, Polymerase Chain Reaction, Bio-optical technology and Immuno sensor have detected volatile breath biomarkers for Mycobacteria. [1, 2, 3, 4, 5, 6, 7] Exhaled nitric oxide (eNO) levels were also analyzed by validated handheld analyzer on culture-confirmed TB cases with a limited value in the direct diagnosis of tuberculosis.[8,9]

The survey of the published literature indicated that desired results with colorimetric sensor array method have not been achieved so far. [10, 11] The basis of detection by colorimetric method using sensor array is the color change that occurs when the analyte interacts with the sensor element. Porphyrins are naturally occurring colored compounds that exhibits interesting photophysical properties which are sensitive towards the environment and are commonly used in a diverse range of research fields. [12, 13,] The colorimetric sensor array of porphyrin has been used to detect various types of organic volatile compounds. [14, 15] This provided us an impetus to utilize the porphyrin sensor array as a tool to detect the organic volatiles exhaled in breath of healthy and diseased person. It is a known fact that the composition of organic volatile compound exhaled will differ as the metabolism in the internal milieu differs inside living body. [16, 17]

It is expected that a unique signature for P-TB patients could be obtained by a proper choice of porphyrin elements that is used to construct the array. If a satisfactory positive predictive value can be obtained even for few porphyrin derivatives, it will open up a new avenue for further studies. Further improvisation can be done by zeroing down on the types of porphyrin compounds to construct future arrays. The interaction properties of the porphyrin can be modulated by variable central metal and peripheral substituent and can be judiciously used to increase the predictive value which is a big advantage for such type of sensor studies. The selectivity in our study with colorimetric sensor was analyzed in pre and post

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exposure to exhaled breath using red, green and blue (RGB) color model based on the Young–Helmholtz theory of trichromatic color vision and on James Clerk Maxwell's color triangle; utilized earlier in cytology extending further to find eosinophilic cells in borderline atypical and malignant squamous cells. [18, 19, 20, 21]

In this study we have embarked on to assess the potential usefulness of qualitative chromatic analysis of exhaled chemicals by porphyrin based colorimetric sensor array and ultimately to identify unique chemical signature of total exhaled human breath of active Pulmonary Tuberculosis (P-TB) cases in comparison with the healthy volunteers. The final aim would be to establish a standard protocol with a cost-effective screening tool.

METHODS

Study participants: The breath analysis was conducted on 62 participants; 32 consecutive P-TB cases presenting at a tertiary care referral state run Sir Thotup Namgyal Memorial (STNM) Hospital; 30 healthy volunteers, free of criteria of P-TB suspect laid down by Revised National Tuberculosis Control Programme guidelines of Government of India (RNTCP), from among the staffs and students of the Sikkim Manipal Institute of Technology and the Sikkim Manipal Institute of Medical Sciences at the Sikkim Manipal University. [22] In both the groups, restrictions on the criteria of sex, ethnicity, smoking or dietary patterns were set aside for participation requisites of inclusion or exclusion criteria.

Study instruments: The 'Breath collection chamber' made of glass was innovatively organized in with compatible vacuum pump (Model: Promivac PVUC-50, HP-17.6, RPM 1440) and a high resolution scanner (Model: HP Scanjet G4050). Twenty-four porphyrin derivatives were synthesized indigenously at the laboratory in Sikkim Manipal Institute of Technology, Sikkim, India under strict supervision and were coded. The quality of the porphyrin derivatives were confirmed by thin layer chromatography techniques to ensure ultra-high purity. The elements were diluted with non-interacting petroleum ether solvent and were spotted onto glass plates from solutions (of a few mM) in thin film spots by dots in construction of sensor; after spotting the colorimetric sensor plates were dried under vacuum at room temperature for thirty minutes before they were in use in our study.

Data collection procedure: The study was approved by Sikkim Manipal Institute of Medical Sciences Ethics Committee (IRB) and Medical Superintendent of the state government run Hospital. P-TB cases were recruited from Chest clinic of the hospital working under RNTCP. Each participant was individually counseled prior to the study that no potential risk was involved in their participation and they had full autonomy to leave the study at any point of time. Each participant provided informed consent. Each individual was explained how they will blow into breath collection chamber by inhaling through nose and exhaling through the disposable tubes into the breath collection chamber. The sensor array was initially RGB scanned and the image of each sensor plate was marked as 'pre-exposure' before placing them inside the breath collection chamber. The chamber containing the sensor plate was made free of the natural constituent of air using high vacuum. The participants were then requested to exhale in the chamber with concurrent, careful slow opening of the connecting stopcock to the chamber; equilibration time of ten minutes was provided to ensure uniform interaction with the sensor elements. Then, the sensor plates were removed from the chamber and immediately scanned and the images marked as 'post-exposure'. To reduce the risk of biohazards, all the precautions were undertaken including use of protection mask by the investigators and single use sterile breathe exhalation tube for the participant that was disposed of using standard operative procedure. Further, the trapped air of one participant was removed from the breathe collection chamber by applying vacuum couple of times before the second participant was approached. The procedure was well tolerated by all the participants with no reported medical complaint; the time required for the exhaled breathe collection did not exceed five minutes that included the counseling times. In total 15 minutes was required for acquisition of final data. This project was undertaken within the range of PhD doctoral research activities under Sikkim Manipal University. The data were strictly kept confidential and were not disclosed for the assessment, management or intervention.

Data analysis: The colour changes of each spot were converted into RGB numerical values with three numeric values per spot from 0 to 255. The data was analyzed in terms of the difference in RGB spectrum value changes in post-exposure state to total exhaled breath in comparison to pre-exposure in each chemical. The sensitivity, specificity, predictive value and likelihood ratio were assessed for each compound in each participant both control and cases in search of a unique signature pattern between healthy and P-TB cases. [23]

RESULTS

In our study, porphyrin array-based chemical sensing showed favorable results for the rapid and direct on spot diagnosis of pulmonary tuberculosis. Overall, the RGB colour spectrum study of the spots changed noticeably after exposure with single total exhaled breath. The different sensor element

interacted differently thus exhibiting varying colour change and is a good indicator for possibility of identifying a unique signature pattern. In RGB analysis, conventionally five or more difference was consistently noted in great majority of the sensor elements. But we compared the RGB differences of ten as cut off point to differentiate exhaled breathe of the healthy and diseased participants. An outstanding difference of responses was observed in seven chemicals between healthy & P-TB cases in the following accuracy parameters:

Sensitivity: Three analytes showed IB, IIBa, ICh highest (86·46%), then IAb and ICd (83·33), IEc (81·72%), IAe (81·25%), ICf (80·21%), IIAc (79·17%). Specificity: Analyte ICa highest (78·89%), then IIAa (65·56%), I (61·11%), IIAb (60·56%). Positive Predictive Value: Analyte ICa (77·11%), IIBa (63·36%), IIAc (62·29%), ICd (62·01%), I (61·11%), IAe (58·65%), IAb (55·94%), ICh (54·97%), IEc (54·68%), IIAa (53·73%) and ICf (53·47%). Positive likelihood ratio: Analyte IIBa 3·45, ICd 2·73, ICa 2·39, IIAc 2·35, IAb, ICh 1·80.

In the binary logistic regression following four analytes showed as fairly strong components in our sensor array: IIBa, ICa, IB, IAb

DISCUSSION

Exhaled breathe analysis in disease detection has historically taken two major directions. The first approach uses spectroscopy and other methods to recognize the individual volatile and non-volatile organic compounds that are remarkable in the exhaled breath. [7, 24] The second path is an advanced procedure with the colorimetric sensor array to find pattern of changes based on the entire blend without qualitative or quantitative detection of specific volatile and nonvolatile components as diseases biomarkers in the exhaled breathe. [10, 11, 25]

We have pursued the latter method in our research work with the total exhaled breath analysis with array-based chemical sensing, based on interactions of different porphyrin with analytes and can have future clinical application. Each of the indigenously synthesized porphyrin based chemicals in our series provided evident changes after exposure to total exhaled breath even when higher cut-off at ten RGB difference in all the participants presenting with pulmonary tuberculosis (P-TB) cases.

In our study the difference of post-exposure values with corresponding pre-exposure values of each porphyrin based chemicals in each participant on a single total exhaled breath could distinguish participants with P-TB. In total, seven porphyrin derivatives showed a limited provisional signature pattern which is an excellent benchmark for the next phase of research. In our sensor array, maximum sensitivity of 86·46 percent could be attained, specificity up to 78.89 percent and positive predictive value up to 77·11 percent.

In a breath analysis using gas chromatography and mass spectroscopy in patients hospitalized for suspicion of pulmonary tuberculosis where the pattern recognition analysis identified patients with positive sputum cultures with 82·6 per cent sensitivity. [7] Breath biomarkers were identified by others with 85 percent accuracy in active P-TB with time-slice alveolar gradients, comprising oxidative stress products and volatile metabolites, when sputum culture, microscopy, and chest radiography all positive or negative. [26] Indian scientists are working on human breath to detect early P-TB with an electronic nose technology. [27]

A multicentere research using concentrated breath, automated thermal desorption, gas chromatography, and surface acoustic, noted naphthalene, benzene and alkanes derivatives as biomarkers of P-TB with sensitivity 71·2 per cent, specificity 72 per cent; accuracy was up to 84 per cent in age-matched subgroups. The investigators predicted 13 per cent positive predictive value of P-TB with in a prevalence of five per cent. [28]

Upcoming researches are exploiting other methods. The urease breath test for tuberculosis (UBT) provided the possibility to diagnose TB though biological plausibility is restricted by co-infection with other urease-producing organism. [29] Differential ion mobility spectrometry method assessed VOCs in experimental mycobacterial chronic intestinal infection in animal model with the potential to become a valuable tool. [30]

The strength of our study was that, we synthesized all the compounds with ultra-high purity confirmed by thin layer chromatography and the compounds were diluted with non-interacting petroleum ether solvent to arrive at highest possible understanding? Thirdly, the diagnostic accuracy was assessed to compare with studies based on other methods. Fourthly, though conventional five or more RGB difference is judged important and was consistently noted in great majority of the sensor elements, we used higher cut-offs (≥10) for interpretation as we were dealing with human participants. Lastly, in exhaled breathe analysis the mass spectrometer consumes about 45 minutes, sputum smear takes two days, sputum culture up to three months, whereas our sensor system needed 15 minutes time period for the required outcomes with portability, simplicity of use to suggest as a tool to aid early identification of P-TB cases.[27]

We had several limitations. The sensitivity and specificity of any method is of paramount importance conventionally used as parameters in bio-psycho-social sciences. Our study was limited by the possible lack of expected sensitivity and specificity, predictive value and likelihood ratios; possibly small sample size had limited the statistical power. Secondly, we worked in the empirical methods in the resource poor settings. Thirdly, there was likely referral bias, as all the study was conducted in an urban tertiary care chest clinic. Fourthly, due to administrative reasons we included all the P-TB cases irrespective of the status of diagnosis or therapy. However, broader adoption will solve these potential confounders.

Future directions of this pattern recognition analysis will help us reach a new screening test that is highly selective as well as simple, rapid, inexpensive and non-invasive viable method of diagnosis for tuberculosis. The quality of interaction of porphyrin with VOCs can be controlled by suitable changes in porphyrin macrocycle like peripheral substituent and central metal. This might lead to fine tuning of senor array by identifying responsive porphyrin derivatives with better predictive value that has to be further standardized to achieve unique colour 'chemical signature' pattern. For this goal, the data need to be collected in additional phases for higher reliability and rational development of cost effective high accuracy sensor tool with broader adoption. To obviate all the potential biases large multicenter trial are needed to compare P-TB diagnosis with the "gold standard" of sputum culture. Further we will extend our study to persons suffering from other disorders and multiple co-morbidities with the standardized protocol to demarcate healthy from cases in total exhaled breathe.

To sum up, porphyrin array-based chemical sensing showed promising results for the direct diagnosis of pulmonary tuberculosis in total exhaled human breath were encouraging though all the compounds were not equally responsive. It would be worth evaluating colorimetric porphyrin elements based sensor array as a likely sensor array in direct diagnosis and prognosis of the active P-TB cases. To the horizon of our knowledge, we are yet to find study reported in the published literature from India on the use of porphyrin based sensors for human health and disease. This array-based chemical sensing can be visualized as a futuristic model of non-invasive diagnosis to fix the chemicals of breath. Our encouraging results augment reliability to porphyrin based sensors to find novel and reliable non-invasive clinical diagnosis with a far reaching impact to establish new range of self-administered instrument.

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Ranabir Pal: conception & design of the study; acquisition, analysis and interpretation of data; drafting the article revising it critically for important intellectual content; final approval of the version

Anoop Gurung: acquisition, analysis and interpretation of data; drafting the article revising it critically for important intellectual content; and final approval of the version.

Sangay Doma Bhutia: acquisition, analysis and interpretation of data; drafting the article revising it critically for important intellectual content; and final approval of the version.

Antara Sharma: acquisition, analysis and interpretation of data; drafting the article revising it critically for important intellectual content; and final approval of the version.

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Conflict of interest:

There are no potential, perceived, or real competing and/or conflicts of interest among authors regarding the article and therefore have nothing to disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work.

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