

Gas Chromatography and Mass Spectroscopy Based Detection of Autoinducer-2 Like Molecule in the Circulation of Tuberculosis Infected Cases

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ABSTRACT

AIM: Autoinducers play a vital role in Mycobacterium tuberculosis by mediating the cell to cell communication resulting in the processes like biofilm formation, virulence and pathogenesis. The present study is focused on the isolation and identification of autoinducer-2 (4,5-dihydroxy-2,3-pentanedione) and similar molecules by Gas chromatography and Mass spectroscopy.

MATERIALS AND METHODS: The TB positive and negative sera were screened on the basis of sputum smear microscopy and the in house developed liposome based antibody detection kit. The lipids were isolated from the sera and derivatized. In GCMS analysis, the molecules were injected in split (1/10) or splitless mode, separated in the Rtx-5MS, cross bonded with 5% diphenyl and 95% dimethyl polysiloxane (30m, 0.25mm ID, 0.25µm). The molecules were identified using mass spectrometric database.

RESULTS: The GCMS analysis revealed the presence of 1,5 bis (trimethyl trisilyl)1,5-pentanedione in the sera of the tuberculosis positive subject. The lipid extract of M. tuberculosis cells showed the presence of 4,4 dimethyl 1-phenyl-1,3-pentanedione. However, in either case, these molecules were separated and detected only in splitless mode.

DISCUSSION: The study concludes that Mycobacterium infection leads to the release of some molecules in the circulation, which are similar to the autoinducer-2 molecules which can be used as a diagnostic marker or targeted to inhibit the bacterial colonies in the host tissue.

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Key words: Autoinducers; Glycolipids; Circulatory Lipid Antigens; Quorum Sensing; 2 (4,5-dihydroxy-2,3-pentanedione), 1,5 bis(trimethyl trisilyl)1,5-pentanedione

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INTRODUCTION

Quorum sensing plays a major role in the maintenance and survival of bacterium and also modulates physiology of the bacterium such as biofilm formation, expression of the virulence, and sporulation^[1]. The bacterium senses the environmental changes and induces signaling mechanisms, mediated by a number of soluble signaling molecules, termed as autoinducers. These autoinducers serve like a hormone; the best example is acyl homoserine lactone. These autoinducers are secreted in the external environment of the cells and directly regulate the gene expression of the bacteria. The well understood signaling molecules found in the bacteria are cyclic adenosinmonophosphate,

cyclic guanosine monophosphate, and guanosine bis pyrophosphate (ppGpp). The cAMP is known to regulate the carbon metabolism while the ppGpp regulates the bacterial survival during starvation^[2,3]. Cyclic-di-GMP is associated with the regulation of responses relevant to pathogenesis, such as motility, secretion, cytotoxicity, and biofilm formation^[4,5]. The regulation of virulence gene in *Vibrio cholerae*, *Salmonella typhimurium* and *Pseudomonas aeruginosa* was reported to be mediated by C-di-GMP signaling^[6,7]. Another metabolite 4,5-dihydroxy-2,3-pentanedione (DPD), called as universal autoinducer (AI-2), was identified to be produced by LuxS synthase. This AI-2 is very specific for a particular bacterium. The *Mycobacterium* encounters the responses of the immune responses like release of reactive oxygen species and reactive nitrogen species^[8-11]. Every bacterial species diffuses very specific AI-2 metabolites which bind to only specific receptors leading to the intracellular signaling in the cues^[12,13]. These signaling mechanisms facilitate the bacterial growth and adaptability in the environment. In a pool of resident and transient microbial community a wide variety of the AI-2 molecules are being secreted, however, the bacterial community can have affinity only for its specific autoinducers. AI-2 mediated signaling process functionally benefits the microbial community to resist the adverse environmental changes, like immune responses more specifically macrophage processing. *Mycobacterium tuberculosis* has been considered as one of the most infectious pathogen. This bacterium has different phases in infection cycle; the phases are adherence to the host cells, entry inside the macrophages, escape from the macrophage processing (immune evasiveness) and development of latency. The *Mycobacterium* sp. apparently survives with its community only with the help of several metabolites, which are secretory or membrane bound. Our report is an initial attempt to identify the quorum sensing molecules present in the circulation of tuberculosis subjects. These molecules were identified using Gas chromatography and Mass spectroscopy and with the aid of several mass spectral databases.

MATERIALS AND METHODS

The present study uses Gas chromatography and Mass spectrometric approach to identify the presence of circulatory pentanedione like molecules in tuberculosis infected subject. The pathology department of the health care centers was contacted personally, and the blood specimen and sputum usually referred for the AFB sputum smear microscopy, culture and other biochemical tests were identified. The sera of AFB positive ($n = 10$) were alone collected and the related information of the cases was obtained from the primary health centers and sera of healthy control ($n = 10$) served as reference. The sera were further reconfirmed using the inhouse developed liposome based antiglycolipid antibody detection TB diagnostic kit. This diagnostic kit carries liposome molecules made of phosphatidyl choline and cholesterol and the cocktail of glycolipid antigens from *Mycobacterium tuberculosis* H37Rv. *M. tuberculosis* H37Rv was cultured in Middle brook 7H9 medium with ADC supplement and the glycolipids were extracted from the *M. tuberculosis* and inserted into the liposome^[14].

The lipids were extracted with chloroform-methanol (2:1) (v/v) and the mixture was agitated in an orbitek shaker at 70 rpm for 16 hours. The organic extract was filtered through 0.2 μ syringe filter. The lipid extract was evaporated to dryness. The lipids were derivatized with 20 μ L of -O-bis (trimethylsilyl)-trifluoroacetamide (BSTFA) (Fluka) for GCMS analysis. The derivatized lipid samples were analyzed through Gas Chromatograph/Mass Spectrometer (Shimadzu QP-2010 plus

with Thermal Desorption system TD 20). The column used was Rtx-5MS, cross bonded with 5% diphenyl and 95% dimethyl polysiloxane (30 m, 0.25 mm ID, 0.25 μ m) with minimal bleed at a temperature ranging 3500C- 330C. The column temperature was conditioned at 1,500 C and injection temperature was optimized at 2,600 C.

The carrier gas helium was purged at a flow rate of 16.3mL/min and column flow was maintained at 1.21 mL/min. The column oven was programmed at 1500C (hold time; 5 min) at 150C/min to 3,100 C (hold time 8 min). The sample injection was programmed at split (1/10) or non split mode, the run time was set to 25 min. The analytes were analyzed through FTD detectors. The mass spectrum of the molecules were analyzed through QP 2010 plus. The data acquisition of the molecules was programmed from 5-23.6 min. The ion source temperature was equilibrated to 2300C, and the interface temperature was maintained at 2800C. The data acquisition at a speed of 1250 with a rise time of 0.5 sec was programmed. The mass fragmentation acquisition was set at a range of m/z 40– m/z650. The mass spectrometric results were recorded and identified using the mass spectrometric databases WILEY and NIST. The presence of tuberculosis specific molecules was further identified through TBDB. The functional information of the molecules was obtained from Human metabolome database (HMDB).

RESULTS

The lipids were isolated from the sera confirmed for tuberculosis and healthy controls. The TB positive specimens were collected from the pathology department of primary health centres. The tuberculosis was confirmed by sputum smear microscopy and the inhouse developed liposome based antibody detection kit. The liposome developed anchors the cocktail of glycolipid antigens isolated from *M. tuberculosis* H37Rv. The bacterial load status and the agglutination results of the TB positive cases and the control were given in the Table 1.

The separation of pentanedione molecule was done using Rtx-5MS, cross bonded with 5% diphenyl and 95% dimethyl polysiloxane (30 m, 0.25 mm ID, 0.25 μ m). Helium was used as a mobile phase. The pentanedione molecules are strongly acidic and we found that 1-phenyl 1,3- pentanedione molecule had a retention of 7.62 min whereas 1,5 pentanedione showed a retention of 9.15 min. The splitless mode of sample injection only favoured the detection of pentanedione molecules. 1.21 mL/min flow of helium was maintained in the column.

The Gas chromatography and mass spectroscopy analysis showed autoinducer-2 like molecules in the tuberculosis infected specimens whereas the same was undetectable in the healthy controls. This molecule had a typical mass fragmentation of m/z 73, 115, 133, 147, 173, 188, 244 and had molecular weight of 244 daltons. Based on the retention index and the mass fragmentation pattern it was identified that this molecule had similarity with 1,5-pentanedione. The GCMS of *Mycobacterium tuberculosis* cell lipid extract showed the mass fragmentation pattern of m/z 27, 41, 51, 69, 77, 91, 105, 120, 128, 147, 161, 176, 189, 204 and a molecular weight of 204 daltons and the molecule was identified as 4,4 dimethyl 1-phenyl-1,3-pentanedione (Figure 1).

The pentanedione molecule, which is considered as an autoinducer molecule, had two hydroxyl groups, however, our findings showed a pentanedione molecule with a phenyl ring attached to the main chain in the *M. tuberculosis* lipid extract and 1,5- pentanedione contains two ketone groups in 1st and 5th positions. Pentanedione molecule with attached phenyl ring in case of the *M. tuberculosis* cell lipids and 1,

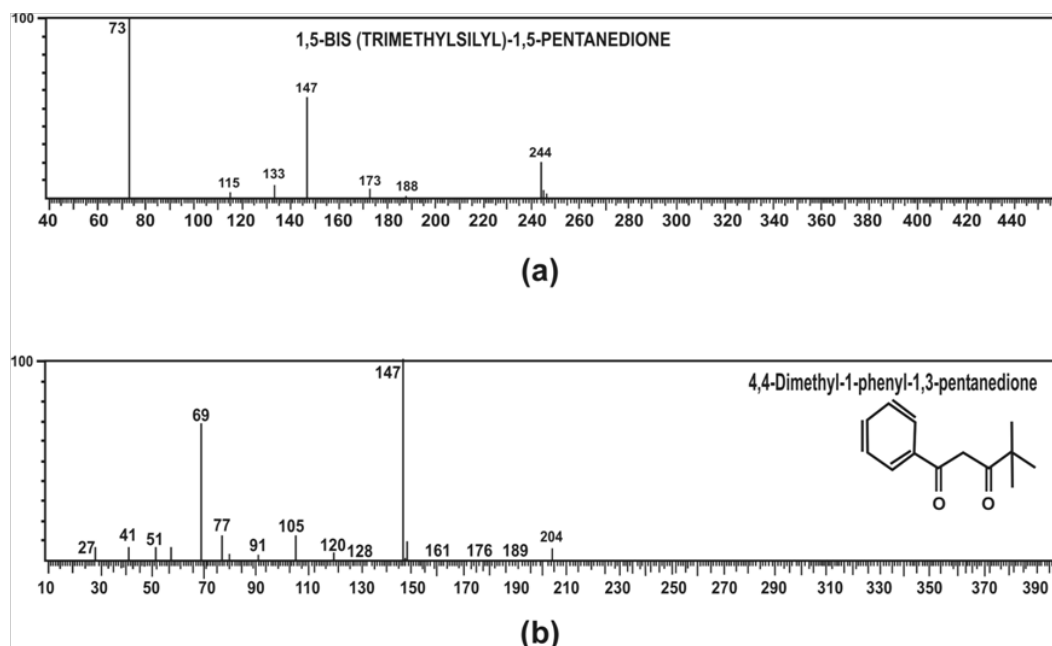


Figure 1 Mass fragmentation of pentanedione molecules. The mass spectrum was matched with the mass spectrometric databases NIST and WILEY.

(a) Mass spectroscopy of TB positive sera specimen The molecule had a Formula: $C_{11}H_{24}O_2Si_2$ and MolWeight:244

(b) Mass spectroscopy of Mycobacterium tuberculosis lipid extract The molecule had a Formula: $C_{15}H_{16}O_2$ and MolWeight:204

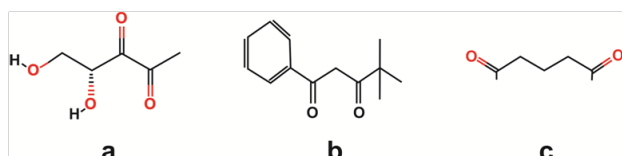


Figure 2 Structural variations of different pentanedione molecules
 a- represents the structure of AI-2 molecules found in Mycobacterium tuberculosis
 b- represents the 4,4 dimethyl 1-phenyl-1,3-pentanedione
 c- represents the 1,5-pentanedione

5- pentanedione contains two ketone groups in 1st and 5th positions (Figure 2).

The overall study revealed that Mycobacterium tuberculosis possesses certain pentanedione molecules in its cells. Similarly, there are certain other pentanedione molecules which were observed in the circulation of the TB infected subjects.

DISCUSSION

The quorum sensing signals identified in bacterium were acyl-homoserine lactones, oligopeptides and autoinducer-2^[15-17]. Some reports reveal that (AI-2) 2, 3-Pentanedione is chemically similar to diacetyl and may have similar toxicological properties while several other reports claim 2, 3-Pentanedione as a natural flavorant and odorant that is also synthesized for use in odor and flavor manufacturing^[18, 19]. This study was intended to identify the autoinducer-2 like signaling molecules in the circulation of tuberculosis infected subject and found that the sputum smear positive and sera showing positivity with liposome based tuberculosis agglutination tests as an aid to detect autoinducer like metabolites. It is well documented that luxS protein is involved in the biosynthesis of autoinducer-2 in *E. coli*, *V. harveyi*, *V. cholerae*, *Enterococcus faecalis* and *Bifidobacteria* having roles in interspecies signaling^[20-22]. Our study showed that autoinducer-2 like

Table 1 Results of agglutination of liposome antigen conjugates with TB positive sera and in different dilutions of sera.

| S. No. | Sputum test | Agglutination test | Dilutions | | |
|--------|-------------|--------------------|-----------|----------|-----------|
| | | | 10 fold | 100 fold | 1000 fold |
| | | | 1+ | N | N |
| TB2 | 2+ | 2+ | 1+ | T | N |
| | | | 2+ | 1+ | N |
| TB4 | 1+ | 1+ | T | N | N |
| | | | 4+ | 2+ | T |
| TB6 | 1+ | 1+ | T | N | N |
| TB7 | 2+ | 3+ | 2+ | 1+ | N |
| TB8 | 2+ | 1+ | T | T | N |
| TB9 | 2+ | 1+ | T | N | N |
| TB10 | 1+ | 2+ | 1+ | T | N |
| C1-10 | - | - | - | - | - |

- TB1-12 indicate TB positive sera clinically confirmed by sputum test.
- C1-10 indicate the normal control sera.
- 1+, 2+, 3+ denote the agglutination strength.
- N - indicate the negative results, T - indicate the trace results.

lipids were detected only at a splitless mode in gas chromatography and mass spectrometer. The splitless injection mode of analysis was performed to avoid the large loss of sample. Earlier reports claim that 2, 3-pentanedione was validated in GC column 30m × 0.25-mm i.d. DB-5 capillary column (df = 0.25 μm) using Helium 1mL mobile phase, the molecule was injected in a splitless mode which is again in agreement with our study. *M. tuberculosis* lipid extract in our study also showed the presence of 4 dimethyl 1-phenyl-1, 3-pentanedione and an autoinducer molecule 4,5-dihydroxy-2,3-pentanedione (DPD). Further, we found 1,5- pentanedione molecules in the circulation of TB infected cases and hypothesized that these signaling molecules would have undergone some chemical modulation inside the host cells. Earlier reports claimed that 4,5-dihydroxy-2,3-pentanedione is highly unstable and undergoes cyclization^[23-25], which is due to the instability of the auto inducer-2 molecule, generated furanones^[26]. However, the exact role of the luxS protein on the conversions of AI-2

molecules is not fully understood^[20]. The concentration of this AI-2 molecule depends on the rate of homocysteine produced and therefore may not be detected in split mode of GC-MS analysis. The overall study revealed that *M. tuberculosis* possesses some molecules which are similar to the AI-2 molecules; however the functional nature of the molecule remains unknown. *M. tuberculosis* showed the presence of autoinducer-2 like molecules on capillary columns crossbonded with 5% diphenyl and 95% dimethyl polysiloxane having capability to separate the autoinducer-2 like molecules. Further, we report here the detection of autoinducer-2 like molecule in the circulation of TB infected cases to act as a biomarker for the detection of active tuberculosis infection, or the pathogenesis of the TB infection. The study is underway to relate the host cell responses to this type of autoinducer molecules.

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CONFLICTS OF INTEREST

The authors declare that they have no competing interests except for the fact that the data has been communicated for patenting.

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