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Original Article

Microbiology Section

Molecular Characterisation of *Stenotrophomonas maltophilia* in Nosocomial Infections: Challenges and Way Forward

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ABSTRACT

Introduction: *Stenotrophomonas maltophilia (S. maltophilia)*, is an important rapidly emerging, opportunistic, non-fermenting Gram negative bacillus with high intrinsic resistance to drugs. It is one of the leading causative agents of nosocomial infections especially in the immunocompromised patients. Molecular typing of pathogens provides an important tool in epidemiological investigations involving nosocomial infections. Due to high geno-diversity, typing of *S. maltophilia* is challenging.

Aim: The study was aimed to evaluate the best epidemiological tool to investigate clonal relatedness of *S. maltophilia*.

Materials and Methods: A prospective study was conducted at a 2400 bedded tertiary care centre in southern India over a period of six months. Twenty-six isolates of *S.maltophilia* were obtained during the study period. Of these, 18 isolates from blood and Endotracheal Aspirates (ETA) cultures were included in the study since they were incriminated in causing nosocomial infection clinically for which appropriate treatment was initiated. These 18 clinical isolates of *S. maltophilia* were characterised to identify the clonality using Conventional Multi Locus Sequence Typing (MLST). A subset of 9 *S. maltophilia* isolates were sequenced using IonTorrent PGM platform. Further phylogenetic analysis was inferred from core genome Single Nucleotide Polymorphisms (SNPs).

Results: Using conventional MLST, one isolate (S04384), was identified as belonging to sequence type 13 (ST13) whereas sequencing of the remaining 17 isolates could not be successfully done using MLST PCR even after several attempts. A subset of nine isolates from these 17 were subjected to sequencing using Ion Torrent PGM platform. Using MLST Finder tool on this platform, one isolate was found to belong to sequence type 15 (ST15). The remaining eight isolates were observed to have novel sequence types; four of which were assigned sequence types ST283, ST284, ST285 and ST286. The remaining four had <50% similarity for *mutM* gene. Further phylogenetic analysis was studied using core genome SNPs. They revealed bifurcating and multifurcating groups among all these nine *S. maltophilia* isolates. None of them belonged to the same clonal group according to SNP based phylogeny.

Conclusion: Frequent recombination events in *S. maltophilia* genome make it difficult to identify the clonality based on MLST. From this study, SNPs based whole genome phylogeny was observed as better methodology to identify clonal relatedness among *S. maltophilia*.

Keywords: Genodiversity, Multilocus sequence typing, Recombination, *S.maltophilia*, Single nucleotide polymorphisms, Whole genome sequencing

INTRODUCTION

Stenotrophomonas maltophilia is an important and increasingly reported nosocomial opportunistic pathogen. The rapid emergence of this organism from being an environmental contaminant to take up causative role in nosocomial infections may be attributed to the wide spread use of broad spectrum antibiotics and chemo therapeutic agents [1]. It has the ability to form biofilms and can colonise medical devices. The common risk factors for infection include prolonged hospital stay, patients on ventilator, underlying malignant condition, use of indwelling catheters, and other chronic immunocompromised states [2]. These pathogens are difficult to treat due to their high level and broad intrinsic resistance [3]. Falagas, after a systematic review, attributed a considerable mortality rate (37%) to *S. maltophila* infections [4]. Organisms like *Acinetobacter*, *S. maltophilia*, MRSA are known to be encountered in outbreak settings. Molecular typing provides an important tool in epidemiological investigations probing outbreak situations when a common source is involved. To identify and characterise the organism as part of nosocomial infection control surveillance is challenging. This is mainly due to high geno-diversity of *S. maltophilia*. It is well known that *S. maltophilia* genome undergoes repeated recombination events resulting in occurrence of novel Sequence Types (STs) and allele types [5]. Multi Locus Sequence Typing (MLST) is a less cumbersome method when compared to other typing methods and the results are available for comparison across different health care settings. But the high recombination frequency often makes it difficult to assign the sequence types and allele types based on seven partial housekeeping gene sequences in MLST. There is no baseline data available on the molecular epidemiology of *S. maltophilia* to date from India. Such data on molecular typing in population structures is important for further studies on the resistance patterns in the region. The possibility of clonal nature in emergence of resistance cannot be overruled [6]. In this study, we made an attempt to evaluate the best epidemiological tool to study clonal relatedness of *S. maltophilia*. This involved conventional MLST (cMLST), whole-genome sequence based MLST (wgMLST) and whole-genome based SNP phylogeny, and their correlation with Antimicrobial Resistance (AMR) profiles.

MATERIALS AND METHODS

The study was conducted in a 2400 bedded tertiary care hospital in southern India, over a six month period from May 2017 to October 2017. A prospective study design was adopted. Clearance was obtained from the Institutional Review Board (IRB) and Ethics

Committee (Ref: IRB No 10915). *S. maltophilia* isolates from blood and ETA samples (with counts >10^5 CFU/mL) causing nosocomial infections in patients admitted to the in-patient wards where treatment was initiated for the same were included in the study. All other isolates of *S. maltophilia* which did not satisfy the above criteria were excluded. A total of 26 isolates were obtained during the study period and 18 of them satisfied the inclusion criteria; eight of them from blood cultures and 10 from ETA.

Identification and AST in the Laboratory

S. maltophilia is a non-fermenting Gram negative bacterium which is oxidase negative. Preliminary screening media consisting of mannitol motility medium, triple sugar iron agar, peptone water and citrate show no change in the medium except for motility being positive. Identification of such colonies which are non-lactose fermenting on MacConkey medium was carried out using Matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) (bioMerieux). Antimicrobial Susceptibility Testing (AST) was performed using disc diffusion as per the recommendations in CLSI guidelines, M100-S27 (CLSI 2017).

DNA extraction: Overnight bacterial cultures grown on blood agar culture plates were emulsified in the sterile saline solution and the genomic DNA isolation was carried out by QiaSymphony (Automated DNA Extraction), as per manufacturer's instructions.

Multi Locus Sequence Typing (MLST)

Conventional MLST (cMLST) was performed using primers and thermal profile conditions for amplification as specified in the PubMLST database (https://pubmlst.org/) using 3500 genetic analyser (Applied Biosystems, CA, USA). Allele sequence identified through cMLST were BLAST matched with NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Whole Genome Sequencing (WGS) and Analysis

Whole-Genome Sequencing (WGS) was performed using IonTorrent PGM (Life Technologies, CA, USA) with 400 bp chemistry for a subset of nine *S. maltophilia* strains (B27164, B23119, B27671, B26854, B09516, S04330, B26847, S04501 and B27675). Sequence types of these tested isolates were identified using allele sequences identified by WGS (wgMLST) using MLST 1.8 tool (https://cge.cbs.dtu.dk/services/MLST/). WGS based SNP phylogeny was calculated using CSI Phylogeny 1.4 tool (https://cge.cbs.dtu.dk/services/CSIPhylogeny/). This Whole Genome Shotgun project was deposited at GenBank under the accession numbers PXIJ00000000, PXIO00000000, PXIL00000000, PXIN00000000, PXIK00000000, PXII00000000, PXIM00000000, PXJF00000000 and PXJG00000000. The version described in this manuscript is version 1.

RESULTS

Patient and Isolate Details

The patients' age group ranged from nine months to 84 years. Pre-existing comorbid conditions in terms of chronic immunocompromised state was seen in all of them and the same is represented in [Table/Fig-1]. Only a single isolate has been taken into consideration for study purpose in cases where *S. maltophilia* was recovered from multiple specimens of the same patient. All blood specimens with *S. maltophilia* were mono-microbial; seven of the ETA had other organisms growing along with *S. maltophilia* in significant numbers (four *Pseudomonas spp*, two *Acinetobacter spp* and one *S. aureus*). All were suspected to have pathogenic potential based on X rays, blood counts, CRP, and clinical presentation and treatment was initiated.

Antimicrobial Susceptibility

As expected all isolates were resistant to carbapenems. The antimicrobial susceptibility profile of the isolates is shown in [Table/Fig-2]. Clinical records showed that 12 (66%) of them received intravenous levofloxacin and the others, trimethoprim-sulfamethoxazole. Fourteen patients showed significant improvement. Two patients were discharged at request due to worsening clinical condition and two succumbed to the infection.

Comorbidity	N	%
Malignancies	5	27
Chronic renal failure	7	38
Chronic liver disease	2	11
*Others	4	22

[Table/Fig-1]: Pre-existing comorbid conditions seen in the study patients. *included congenital heart disease, diabetes, hypothyroidism.

Drug	Resistant (*n*)	Intermediate (*n*)	Susceptible (*n*)
TMT-SMT	1	-	17
Levofloxacin	0	-	18
Ceftazidime	12	-	6
Tetracycline	10	1	7

[Table/Fig-2]: Antibiotic susceptibility pattern observed in the isolates of *S. maltophilia* (n=18).

MLST

To identify the clonal relatedness of these isolates, conventional MLST (cMLST) was performed. For 17 out of 18 isolates, MLST PCR and sequencing was not successful for either one or more genes out of seven house-keeping genes even after several attempts. One isolate, S04384 was identified as ST13 by cMLST.

Whole Genome Sequencing

WGS was performed on a subset of nine isolates from the remaining 17 isolates on which cMLST was not successful. Analysis of WGS results using K-merFinder 2.5 tool (https://cge.cbs.dtu.dk/services/KmerFinder/) confirmed that all nine sequenced isolates were *S. maltophilia*. Sequences were analysed through MLST Finder and revealed the sequence type for one isolate (B27164) to be ST15. Other eight isolates were observed as novel sequence types. Most of the alleles in these eight WGS isolates did not match accurately with the allele sequences from the database (98-99% identity). The alleles with varied sequences were submitted to the PubMLST database to obtain new allele types and sequence types. As a result, four novel sequence types (ST283, ST284, ST285 and ST286) of *S. maltophilia* were identified using whole genome MLST (wgMLST). However, whole-genome SNP phylogeny revealed bifurcating and multifurcating groups among the nine *S. maltophilia* isolates. None of the isolates belonged to the same clonal group as revealed by SNP based phylogeny.

DISCUSSION

Much of the literature on *S. maltophilia* available from India is in the form of case reports. To our knowledge this is the first study employing molecular typing methods on *S. maltophilia* from India. Worldwide many genotypic molecular typing approaches and targets have been evaluated for their discriminatory power to identify the clonal relatedness of *S. maltophilia*; these included ribotyping, DNA-DNA hybridizations, Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphisms (RFLP) of the *gyrB* gene, Pulsed-Field Gel Electrophoresis (PFGE), and Enterobacterial Repetitive Intergenic Consensus Sequence-PCR (ERIC-PCR) [2]. The details of the findings on evaluation of different methodologies on discriminatory ability and robustness of aforementioned methods are given in [Table/Fig-3] [5-16].

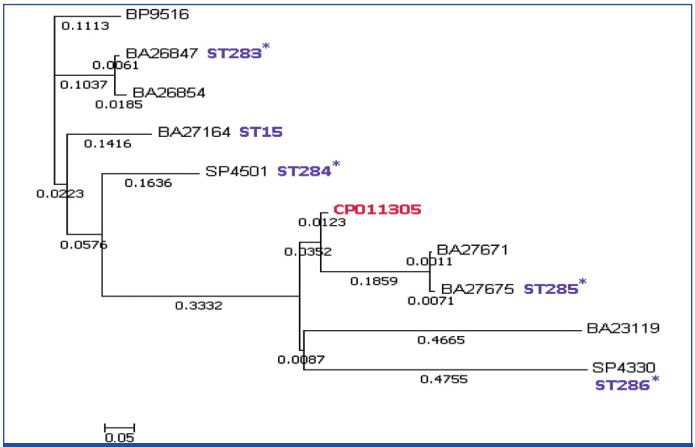
It is evident that surveillance of *S. maltophilia* using MLST platform often assigns novel ST and allele types which makes it difficult to define the clonal groups [5,6]. This is due to high frequency

	Typing method	Discriminatory ability of the methods	Remarks	References
Conventional typing	AFLP	Diversity between environmental and human clinical samples of *S. maltophilia* was observed with variation of 60%	Reproducibility is low Reference available is not standard and lacks global comparison	Hauben L et al., [7]
	RAPD and ERIC-PCR	First use in *S. maltophilia* observed high diversity (76%) among infected patients		Chatelut M et al., [8]; Caylan R et al., [9].
	RFLP	191 isolates revealed 9 clusters with internal similarity >75% Demonstrated the antimicrobial resistance due to changes in *S. maltophilia* strains over a period of 15 months in same patient		Wust J et al., [10]; Coenye T et al., [11]
	PFGE	139 strains formed 5 clusters and 71% of isolates showed dissimilar banding pattern High diversity of strain relatedness in hospital sources is noted	Helpful in defining an outbreak from smaller population i.e., hospital outbreaks Most discriminatory among the conventional methods	Berg Get al., [12]; Valdezate S et al., [13]; Jumaa PA et al., [14]
Sequence based methods	MLST	54 STs observed from 70 strains (77%) Recombination events were detected in one-sixth of the sequence types	Resulted in diverse new sequence/allele types in every analysis	Kaiser S et al., [5]; Cho HH et al., [6]
	WGS	Identified significant genome diversity in the isolates	Accurate identification of allele types using complete sequences	Pak TR et al., [15]
	SNP based phylogeny	Lineages were reported based on 334 polymorphic mutations SNP analysis proved that mutations arising independently in two lineages co-localise in space, providing evidence for location-specific selection in human body	Would help in analysing the closeness of the clinical isolates and in identifying the frequency of recombination events	Chung H et al., [16]

[Table/Fig-3]: Various typing methods with discriminatory ability for clonal relatedness of *S. maltophilia* [5-16].

of recombination events naturally seen in *S. maltophilia* [17]. Interestingly, in this study the allele numbers for few genes assigned by cMLST (uses partial target region) were not correlating with wgMLST (complete allele sequence). Cho HH et al., had reported that out of 33 clinical isolates, 28 isolates exhibited 23 new sequence types (STs), whereas only five isolates exhibited previously described STs (3 STs) [6].

Way forward to the existing limitation is the whole genome based SNP analysis. This might give much better insights into the phylogenies of *S. maltophilia*. Lira F et al., had reported that the complex diversity of *S. maltophilia* formed an interlaced taxon and shared the same attributes between clinical and environmental strains [18]. In this study, SNP based phylogeny revealed that every study isolates is divergent from the other [Table/Fig-4].



[Table/Fig-4]: SNP based phylogeny.

The relation between the AMR phenotypes when compared using cMLST, wgMLST and SNP based typing is given in [Table/Fig-5]. AMR phenotypes showed six different types based on the individual susceptibility profiles. cMLST could not identify any STs. wgMLST identified five STs, of which, four were novel. Whereas, SNP based phylogeny revealed all were different from each other with various root to tip distance values indicating high heterogeneity, except B09516 and B26847, which may be from a single clone source. It can be derived that SNP based method is superior to cMLST and wgMLST. Isolates B26847 and B26854 differ with 0.01 nucleotide substitution per site, however they share the same AMR phenotype, which infers that the nucleotide substitution in the genomes is not related to resistance. Similar results were noted between strains B27671 and B27675.

Isolate ID	AMR Profile (SXT-LEV-CZD-TET-PTZ)	cMLST	wgMLST	SNP phylogeny Root to tip distance (nuc. subst./site)
B09516	CZD-TET-PTZ	-	-	0.1
B26847	PTZ	-	ST283	0.1
B26854	PTZ	-	-	0.11
B27164	-	-	ST15	0.15
S04501	TET-PTZ	-	ST284	0.24
B27671	CZD-TET-PTZ	-	-	0.63
B27675	CZD-TET-PTZ	-	ST285	0.64
B23119	CZD-PTZ	-	-	0.89
S04330	TET	-	ST286	0.9

[Table/Fig-5]: Comparison of AMR profile with cMLST, wgMLST and SNP based typing methods.
SXT: Trimethoprim-sulfamethoxazole; LEV: Levofloxacin; CZD: Ceftazidime; TET: Tetracycline; PTZ: Piperacillin/tazobactam; -Not identified; nuc. subst. /site–nucleotide substitution per site

LIMITATION

The limitation of this study is the small number of isolates tested and the findings need to be validated using a larger number of clinical isolates.

CONCLUSION

The study reveals that cMLST and wgMLST are not reliable methods for typing of *S. maltophilia* strains due to frequent recombination events taking place in its genome. Whole genome SNP based phylogeny may help in the discrimination bringing clarity on the clonal relationship. This accurate phylogeny based on SNPs and protein composition may be the molecular typing method of choice for investigating nosocomial spread in the case of *S. maltophilia*.

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