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A Study of Molecular Characterization of the Trimethoprim Resistant *Salmonella typhi* Strains Prevalent in Himachal Pradesh

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Abstract

Typhoid is a major public health problem in tropical and subtropical countries including India. Salmonella typhi, the causative agent of typhoid fever, is a gram-negative, motile, rod shaped, facultative anaerobe. It is solely a human pathogen and there is no animal reservoir. Antibiotic therapy is the mainstay for the treatment of typhoid fever and the complications associated with it. Nowadays, emerging multidrug resistance among Salmonella typhi strains has become a major public health problem. Present research work was carried out for the identification and molecular characterization of Trimethoprim resistant Salmonella enterica serovar typhi strains from individuals suffering with typhoid fever by means of various techniques i.e.; biochemicals, phenotypical and drug resistant gene specific polymerase chain reaction (PCR). A total of 14 blood specimen of infected patients were collected from Solan district of Himachal Pradesh with varying age groups and were processed via broth enrichment methods for primary isolation and identification of typhoid bacilli. Microbiological and biochemical investigations revealed the presence of S. typhi in all 14 specimens. The antibiotic susceptibility assay was carried out for 11 antimicrobial to study the MDR pattern of the identified bacilli. It was observed that 14/14 S. typhi strains were 100% resistance to Trimethoprim, Co-trimoxazole, Sulfanilamide, Penicillin, Ampicillin, Oxacillin, Tetracycline and Erythromycin, and 70-100% susceptible to Levofloxacin, Amikacin and Amoxicillin. The PCR analysis of these MDR strains showed the presence of dhfr a7 (365 bp) gene in only 10/14 isolates. This study confirmed that Trimethoprim resistance in these strains were due to the presence of dhfr a7 gene and also that PCR based diagnosis could be very useful for the rapid detection of drug resistant S. typhi strains. Present study emphasize that Trimethoprim drug is no longer useful for the treatment of typhoid fever as its MIC optimization was very high (750µg/ml). This study may further help the researchers in selecting the appropriate therapeutic approaches targeting Trimethoprim resistant Salmonella typhi strains.

***Keywords**: S. typhi – Salmonella typhi, dhfr- dihydrofolate reductase, MDR- multidrug resistance, MIC- multi drug resistance, Trimethoprim and Typhoid fever*

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1. Introduction

Typhoid fever is distressingly prevalent in developing countries, where it remains a major public health problem. The annual global incidence of this disease has been estimated to be 21 million cases, with more than 700,000 deaths [13]. Infection with *Salmonella typhi*, the causative organism of typhoid, requires effective antimicrobial chemotherapy in order to reduce morbidity and mortality. Since 1989, various multidrug-resistant (MDR) *S. typhi* strains which are no longer susceptible to the first-line antibiotics have emerged [8]. Indeed, these MDR *S. typhi* strains have become a serious problem globally and have been reported not only in the Indian subcontinent but also in Latin America, Egypt, Nigeria, China, Korea, Vietnam, and the Philippines. Trimethoprim is an antibiotic or synthetic antibacterial agent that target naturally occurring enzymes, degrading or modifying them are unlikely. Trimethoprim (TMP) inhibits bacterial dihydrofolate reductase (DHFR) enzyme activity by competitively binding to the substrate binding site. Trimethoprim was once a safe and cost effective drug widely used in combination with sulfa drug to treat typhoid. The emergence of resistance against Trimethoprim has been reported from several part of the world including India [24].

The drug resistance to Trimethoprim is plasmid mediated which emerged in Gram-negative bacteria like *S. typhi* within a few years of the clinical introduction of the drugs [24]. Shortly after the clinical uses of TMP, a new family of plasmid mediated DHFRs appeared among enterobacteria which had TMP-insensitive target sites [25]. The different plasmid-encoded DHFRs families belonging to the incompatibility complex group *Inc*HI [8] often mediate moderate- to high-level of resistance to TMP. These *Inc*HI plasmids are large (~180 kb), conjugative and originated from Southeast Asia [7, 18]. So it is the need of hour to study the molecular characterization of this drug resistant mechanism developed by the bacilli and the ideas to overcome these situations. The present study was aimed for the identification and molecular characterization of the genes responsible for the Trimethoprim resistance in *S. typhi* strains.

2. Materials and Methods

2.1. Microbial Strains

The Standard strain of *Salmonella typhi* (MTCC-733) was obtained from IMTECH, Chandigarh and 14 clinical blood specimens were collected from regional hospital Solan and processed in Molecular and Immune-parasitology research laboratory, Faculty of Applied Sciences and Biotechnology, Shoolini University, Solan, H.P.

2.2. Ethical Considerations

The project has been approved by Institutional Ethical Committee of Shoolini University. Written informed consent or approbation was obtained from patients or guardians of children before inclusion in the study.

2.3. Characterization and Identification of *S. typhi* Strains

These strains were characterized by comparing to standard strain on different enriched and specific media. All the clinical isolates were identified by staining and biochemical tests.

2.4. Antibiotic Susceptibility Assay

All 14 isolates and standard strain were cultivated in specific media and screened for susceptibility/resistant pattern against 11 antibiotics (i.e., Trimethoprim (Folate inhibitor), Co-trimoxazole (Folate inhibitor), Sulfanilamide (Folate inhibitor), Penicillin (β lactams),

Ampicillin (β lactams), Oxacillin (β lactams), Tetracycline (Tetracyclines), Erythromycin (Macrolids), Levofloxacin (Fluoroquinolones), Amikacin (Aminoglycosides) and Amoxicillin (β lactams)) according to Kirby-Bauer method to identify MDR strain.

2.5. Plasmid DNA Isolation and Visualization

The resistant strains were grown in Trimethoprim treated medium for the extraction of plasmid DNA by alkaline lysis method as described by Takahashi, S. and Nagano, Y. [29]. The plasmid DNA was electrophorased on 0.8% Agarose gel stained with Ethidium Bromide (0.5µg/ml) in 1X TAE buffer, pH 7.8 (40mM Tris base, 20mM Acetic acid and 1mM EDTA). 5µl of plasmid DNA was loaded on Agarose gel. The constant voltage of 100V for 1 hour was applied and the product was visualized and photographed using gel documentation system (UVP Cambridge, UK. Gel Doc-IT ^{R} 310 imager).

2.6. PCR Amplification and Visualization of *dhfr a7* Gene

The amplification of the target gene from plasmid DNA was carried out in a thermal cycler (Applied Biosystems Veriti 96 well thermal cycler). A 50µl reaction volume comprising of 1X PCR reaction buffer, 0.2mM dNTPs and 1.25U of Taq DNA polymerase enzymes was processed in thermal cycler by using gene specific oligonucleotide primers as described by Schwalbe *et. al.*, [26]. DHFR7-F-5′GGTCCAACCCATTGCTTTAC3′; DHFR7-R-5′CACGGAAAGAAATCACAAC3′. The PCR amplified products were electrophoresed on 1.2% Agarose gel stained with EtBr (0.5µg/ml) using the appropriate DNA molecular weight marker and visualized under UV transilluminator and photographed.

3. Results and Analysis

The culture characterization, staining and biochemical analysis of all the 14 specimens as compared to standard strain, confirmed the isolates as *S. typhi*. The susceptibility assay of 11 antimicrobial agents against *S. typhi* showed 100% resistance to Trimethoprim, Co-Trimoxazole, Sulfanilamide, Penicillin, Ampicillin, Oxacillin, Tetracycline and Erythromycin, and 70-100% sensitivity to Levofloxacin, Amikacin and Amoxicillin (Figure 1). The minimum inhibitory concentration of Trimethoprim drug against *S. typhi* strains were obtained by microtitre plate dilution method. The MIC obtained by this method was very high (750µg/ml) which states that all the 14 *S. typhi* isolates were highly resistant to trimethoprim drug. All the results were analyzed using statistical formulas of mean and standard deviation.

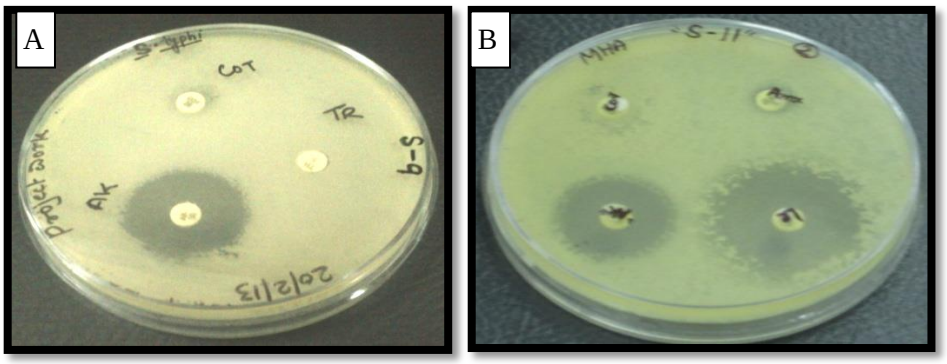

Figure 1. Showing the Efficacy of Antibiotics: A) Resistant to Co-trimoxazole, Trimethoprim and Sensitive to Amikacin. B) Resistant to Amoxycillin, Co-trimoxazole and Sensitive to Levofloxacin and Amikacin

Approximately 120 kb plasmid was observed under UV transilluminator which harbor Trimethoprim resistant *dhfr A7* gene. The RepHIA region of *dhfr A7* gene was amplified by using specific primers. Out of 14 isolates PCR product was obtained in 10 (71.4%) isolates only. Remaining samples were not amplified even after repeated attempts. PCR amplified product does not show any variation on Agarose gel (Figure 2). The molecular weight of RepHIA region of *dhfr A7* gene obtained in PCR product was 365 bp for all isolates (Figure 2). The results show that only 10 (71.4%) isolates carry the *dhfr a7* gene which was resistant for the Trimethoprim antibiotic whether the others not. The Trimethoprim resistance in 4 strains might be due to another factors or perheps RepHIA region of *dhfr a*7 gene could not amplified due to the mutation in this region.

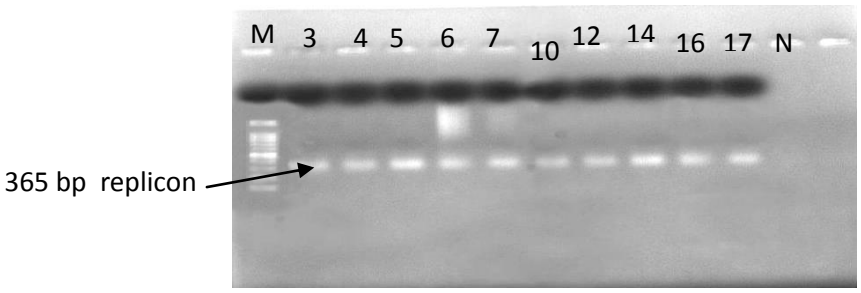

Figure 2. PCR ampLification of 365 bp region of RepHI1A Replicon of *dhfr A7* geNe (LANE; 3-17) of 10 Isolates of *Salmonella typhi*, M = 100 bp DNA Marker, N = Negative Control

4. Discussion

Typhoid fever is a major public health problem in tropical and subtropical countries including India. The development of MDR strains in causative agent *S. typhi* is an alarming situation in endemic region. There has been increasing concern about the prevalence of MDR *S. typhi* strains insusceptible to chloramphenicol, ampicillin, and trimethoprim [19]. Indeed there is an urgent need to study the status of drug resistant *S. typhi* strains prevalent in any geographical region so that a rational approach to therapy may be adopted. Infected and healthy carriers were the source of infection and "five Fs" (food, fingers, flies, fomites and faeces) played an important role in the spread of the disease [5]. In the present study, 14 isolates of *S. typhi* were obtained from regional hospital Solan, HP from individuals below 30 years of age in which attack rate is significantly higher.

Similar to the present study, the detection of typhoid bacilli from the patient of less than 30 years of age was also reported from Tamil Nadu in high frequency [1]. In PCR analysis, *dhfr a7* gene was targeted for the Trimethoprim resistance based identification of *S. enterica* serovar *typhi* which revealed the detection of several isolates originated from typhoidal humans. This study demonstrated that the gene was predominant in the isolates of *S. typhi*, which could be used as specific marker gene for the rapid detection of *S.typhi*. The present study suggested that the *S. typhi* strains prevalent in this region might be multiple drugs resistance. In accordance to our study, the co-existence of antibiotic-sensitive and MDR strains of *S. typhi* was reported from South Korea [28], the author also reported the presence of Plasmid in MDR strains while it was not extracted from sensitive strains. Similarly, the presence of Plasmid in MDR strains of *S. typhi* and absence of plasmid in the sensitive strain has also been reported from Kolkata, India [27]. The R-plasmid is an unstable plasmid that may appear or disappear at any time resulting in the emergence of drug resistant or drug sensitive isolates. The selection exerted by antibiotic treatment of enteric fever may be the cause of acquisition of R- plasmid [21]. Through the acquisition of a plasmid conferring multidrug resistance, the strain undergoes the necessary and appropriate adaptation for survival in the changing antibiotic environment.

The present study revealed the PCR amplification of dhfr A7 in isolates suggesting the presence of R-plasmid and trimethoprim resistance gene in these isolates which is accordance to the earlier study reported in MDR strains. The product of novel trimethoprim resistance gene dhfriVII was found closely related to type I, type V, and type VI dihydrofolate reductases (64 to 71%). Also these enzymes are 32% identical to the chromosomal enzyme of E. coli [6] and 29% identical to the human enzyme [10]. The similarity between the members of this family of plasmid- encoded dihydrofolate reductases helps to understand the relationship between amino acid structure and antifolate inhibition. The binding of trimethoprim to the enzyme from E. coli was studied by Matthews et. al., [4]. The nature of the amino acid residue plays important role for the resistance to this drug. The plasmid-borne dihydrofolate reductases originate from the chromosomal enzymes of other microorganisms which possibly carrying the integron effected the horizontal transmission of these genes to enteric bacteria, in which their natural ability to cause resistance has been utilized.

According to Sundström et. al., [15] the dhfr VII gene starts with a UUG codon and dhfrI and dhfrV both start with GUG codons suggests that these genes did not originally come from members of the family Enterobacteriaceae. The transposon-like element named an integron seems to be an efficient carrier of trimethoprim resistance genes. Among the many antibiotic resistance-mediating cassettes found in integrons are several trimethoprim resistance genes. These are dhfrI in pLMO150 and pLMO229 [14], dhfrIb in R388 and dhfrV in pLMO20 [16], dhfrIIa in R67 and dhfrIIc in R751 [22], and dhfrVII in TnSO86 [15]. The dhfrVI gene of pUK672, recently described by Wylie and Koorn hof [3], has not been identified as an integron cassette, but the presence of the characteristic repeats around the gene strongly suggests that it is indeed part of a cassette. The original location of dhfrI in transposon Tn7 [23] also turned out to be in a cassette, identical to that of dhfrI in the integrons of pLMO150 and pLMO229 [14, 17].

The DHFRs have high degree of homology in the conserved regions, it is vital that specific oligonucleotide probes be used in order to distinguish between the different dhfrs [24]. The identification of the plasmid-encoded type VII DHFR in S. typhi confirms the ubiquitous distribution of this particular DHFR. This enzyme has already been isolated in Sweden, Finland, Nigeria, Sri Lanka, the United Kingdom, and in South Africa [15]. Since 1989 outbreaks caused by strains of S. typhi resistant to Trimethoprim and with additional resistance to streptomycin, sulfonamides, and tetracycline have been reported in many developing countries, especially Pakistan [12] and India [11]. Such strains have been termed multidrug-resistant (MDR). MDR strains have also caused outbreaks in Bangladesh [20], Southeast Asia [9], North Africa [2], and South Africa [30].

The present study revealed the prevalence of S. typhi strains resistant to Trimethoprim followed by co-trimoxazole and sulfanilamide resistance in Solan district of Himachal Pradesh. The emergence of MDR strain in this region has become a major problem for public health, which needs consideration to control and prevent this disease in Solan district. Although, the data of this study is preliminary and a large scale study is needed to be undertaken.

5. Conclusion

This is concluded from the present study that MDR strains have become an alarming problem in the poor sanitary areas. The antimicrobial Trimethoprim, which was once used as a drug of choice can't be prescribed for the therapeutic measures of typhoid due to increasing worldwide spread of Trimethoprim resistant *Salmonella* strains. The present study suggested that a close surveillance of these strains is essential to monitor their spread, antimicrobial resistance profile, and association to the disease. This study may further help the researchers to select appropriate therapeutic approaches targeting

Trimethoprim resistant *Salmonella typhi* strains and in the development of some naturally derived prevention measures.

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