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Bacterial profiling of healthy bone marrow using polymerase chain reaction technique

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Abstract:

BACKGROUND: The bone marrow of healthy individuals is conventionally considered sterile like other body fluids, but recently the scientists found genetic material from the bacteria inside their stem cells. The findings raise the possibility that other infectious agents may also reside in the bone marrow.

OBJECTIVES: The study was planned to look for the presence of all type of bacteria by amplifying 16S rDNA sequences using eubacterial universal primers.

MATERIALS AND METHODS: A total of 18 bone marrow samples of apparently healthy individuals were collected from patients admitted having closed bone fractures. The DNA was isolated and was subjected to nested polymerase chain reaction (PCR) using Universal eubacterial 16S rDNA primers. The samples positive by universal PCR was further checked for the presence of *Salmonella* Typhi, *Salmonella* Paratyphi A, and/or *Mycobacterium tuberculosis* (*Mtb*) if any using a second nested PCR reaction.

RESULTS: A total of 16 (89%) samples could yield the desired amplicon through universal PCR. The secondary PCR of 16 samples, the desired amplicons were detected 3 (18.8%) for *Mtb*, 4 (25%) for *S. Typhi*, and 1 (6.2%) for *S. Paratyphi A*.

CONCLUSIONS: Even in asymptomatic cases, other infectious agents such as *Mtb*, *Salmonella* spp., and many other microorganisms may be present as commensal.

Keywords:

Bone marrow, *Mycobacterium tuberculosis*, polymerase chain reaction, *Salmonella* Paratyphi, *Salmonella* Typhi A

Introduction

The bone marrow constitutes about 5% of total body weight in humans.^[1] It is an interesting fact that most of the pluripotent stem cells reside in it. Conventionally, bone marrow of healthy individuals is considered sterile like other body fluids, i.e., blood, pleural, peritoneal, pericardial, cerebrospinal, and joint fluids. Nevertheless, there is an intriguing article entitled "CD271+ bone marrow mesenchymal stem cells (BM-MSCs) may provide a niche for dormant *Mycobacterium tuberculosis*," mentions that the CD271+ stem cells are self replicating, resides in secured niches

in the bone marrow virtually inaccessible to the immune system, and are largely impervious to drug molecules.^[2] For much of their life cycle, the stem cells stay in the bone marrow, eventually emerging into the bloodstream to circulate throughout the body. The authors suggest that bone marrow might be the safest place of such facultative and obligate intracellular parasite and thus causing the chronicity of the infection.^[3] Interestingly, DNA of a wide variety of organism had been isolated from the bone marrow of the patients who had suffered from chronic diseases like leishmaniasis, brucellosis, etc., after they had been declared clinically cured, even in immunocompetent patients.^[4,5]

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Our laboratory has carried out work related to screening for different bacteria in bone marrow of patients suffering from aplastic anemia and hematological malignancies (AML, CML, ALL, CLL, etc.). We have observed the presence of *Salmonella species* and *Mtb* in bone marrow collected from children suffering from aplastic anemia and also from cases of hematological malignancies (unpublished data). Further, we could detect *Mtb* in a patient suffering from chronic osteomyelitis also. Very high prevalence of these individuals prompted us to carry out the present study, speculating that bone marrow is not an organ free of microbes.

Thus, it is prudent enough to question the conventional ideology which vehemently accepts the absolute sterility of bone marrow in healthy persons. The possibility of commensal bacteria cannot be negated without carrying out the metagenomic approach and also looking for known pathogenic bacteria in the bone marrow. With this aim, we planned the present study to look for the presence of all type of bacteria by amplifying 16S rDNA sequences using eubacterial universal primers. Further, to identify the spectrum of known pathogenic bacteria, we planned to use specific nested primers.

Materials and Methods

Ethical consideration

The study plan was approved by the Institute Ethics Committee of Banaras Hindu University, Varanasi, and informed consent was obtained from each of the participants/guardians.

Study design

The present study is an observational study, carried out in the Department of Microbiology and the Department of Orthopedics Institute of Medical Sciences, Banaras Hindu University, Varanasi, India. The total duration of the study was 2 years extending from August 2014 to July 2016. In the study, a total of 100 traumatized patients admitted to the Orthopedics department having closed bone fractures were screened as per the inclusive criteria of our study. Out of these, a total of 18 nonduplicate bone marrow samples of apparently healthy individuals were collected from patients. The bone marrow was drained aseptically during surgery from the cases that were planned for elective surgery.

Patients on medication causing bone marrow suppression, pregnancy, seropositive for hepatitis B surface antigen, anti-HCV, HIV, anemia of chronic diseases, and patients with a present or past history of any chronic infectious disease were excluded from the study.

Collection, storage, and processing of the specimen

Bone marrow aspirated (about 15 ml) from exposed bone marrow of patients with full aseptic precautions in operation theater was sent to the Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University in a plane sterile vial. Out of this, 10 ml of sample was inoculated in Brain Heart Infusion broth and incubated at 37° C. The broth was subsequently subcultured on blood agar at 7th day to look for any bacterial growth. The remaining volume of samples was stored at -20°C temperature till further use.

Molecular study

Extraction of DNA

DNA isolation was done using standard, phenol-chloroform method as described by Sambrook *et al.*^[6]

Polymerase chain reaction primers

Bacterial rDNA consists of highly conserved nucleotide sequences that are shared by all bacterial species, interspersed with variable regions that are genus or species specific. Primers based on the conserved sequences of the 16S rDNA gene, ubiquitous in bacteria, were used to detect the presence of bacterial DNA in bone marrow samples [Table 1].^[7]

Heat shock protein gene (*hsp65*) of *Mtb* is known to be quite conserved, and in-house designed well-tested primers *hsp TBF1* and *hsp TBR1* were selected for amplification of a 494 bp nucleotide sequence for primary polymerase chain reaction (PCR); *hspTBF2* and *hspTBR2* were selected for amplification of a 218 bp nucleotide sequence for nested PCR [Table 1].^[8]

Oligonucleotide primers for *Salmonella Paratyphi A* were synthesized from the sequence of putative fimbrial protein (*stkG*) gene sequence Accession No. CP000026; GI: 56126533. Oligonucleotides *stkG F1* and *stkG R1* were used in the first round PCR to amplify a 427 bp fragment which corresponds to nucleotides 96118 and 522-501, respectively [Table 1]. For nested PCR, oligonucleotides *stkG F2* and *stkG R2* were used from amplified product of first round PCR to amplify a 229 bp, correspond to 138159 and 366-343, respectively, of putative fimbrial protein (*stkG*) gene of *S. Paratyphi A* as described by Pratap *et al.*^[9]

For the detection of *Salmonella Typhi*, primers were used targeting flagellin (*fliC*) gene sequence as described by Song *et al.* and modified by Frankel [Table 1].^[10,11] The first round PCR of both genes was amplified 495 and 537 bp, whereas nested PCR amplified 364 and 377 bp, respectively, from amplified product of first round PCR.

Taq polymerase enzymes and customized primers were procured from SBS Genetech Co., Ltd., China.

Table 1: Primers and amplification conditions targeting *Mycobacterium tuberculosis*, *Salmonella Paratyphi A*, and *Salmonella Typhi*

Target gene	Cycle	Primer names and sequences	Amplicon size	Annealing temperature and number of cycles	Reference
16S rDNA (Universal eubacterial primers)	Primary cycle	16S F 5'-TTG GAG AGT TTG ATC CTG GCT C-3' 16S R 5'-ACG TCA TCC CCA CCT TCC TC-3'	1194 bp	56°C for 30 s for 30 cycles	Okhravi, et al., 2000 ^[7]
	Nested cycles	NF 5'-GGC GGC AKG CCT AAY ACA TGC AAG T-3' NR 5'-GAC GAC AGC CAT GCA SCA CCT GT-3'	1025 bp	56°C for 30 s for 30 cycles	
		Heat shock protein 65 (<i>hsp65</i> or <i>groEL2</i>)	Primary cycle	hspTBF1 5' AAA AGC CGG ATG GCA ATT CG 3' hspTBR1 5' ATT ACC GGC TTG GAC CCC CTG 3'	
Nested cycles	hspTBF2 5' ATG CGG CGC AGA CCG AAG ACAAG 3' hspTBR2 5' TCA ACG CGC TGT CTA GCT TGT C 3'	218 bp	65°C for 1 min 35 cycles		
Putative fimbrial gene (<i>stkG</i>)	Primary cycle	<i>stkG</i> F1 5' CGTTACTGAGGTACAGGCATC-3' <i>stkG</i> R1 5'-CACATTGTTCTCGGAGACCCCA-3'	427 bp	51.5°C for 30 s for 30 cycles	Pratap et al., 2014 ^[9]
	Nested cycles	<i>stkG</i> F2 5'-CAATGGCTTCTGGCGAACTGTC-3' <i>stkG</i> R2 5'-TGGAGAAAGATCAGACCACCGAG-3'	229 bp	51.5°C for 30 s for 30 cycles	
		Flagellin gene (<i>fliC</i>)	Primary cycle	ST1 5'-ACTGCTAAAACCACTACT-3' ST2 5'-TTAACGCAGTAAAGAGAG-3'	
Nested cycles	ST3 5'-AGA TGG TAC TGG CGT TGC TC-3' ST4 5'-TGG AGA CTT CGG TCG CGT AG-3'		364 bp	51.5°C for 30 s for 30 cycles	

T = Thymine, A = Adenine, G = Guanine, C = Cytosine

Polymerase chain reaction amplification of bacterial 16S rDNA

The DNA isolated from the bone marrow was subjected to amplification using Universal eubacterial 16S rDNA primers [sequence as Table 1]. PCR reaction was carried out in 25 µl volume. Reaction mixture contained 10 × reaction buffer (5 µl/sample), dNTPs (0.25 mM each), forward and reverse primers (10 pmole each) and *Taq polymerase* enzyme (1 unit/reaction), and 1.5 mM MgCl₂. The template used was ranged between 50 ng and 100 ng of DNA from each of the patient samples. Amplification was carried out on BIO-RAD system with a heated lid. The hot start method was employed by heating at 94°C for 5 min initially. Thereafter, amplification was carried out for 35 cycles at 90°C for 1 min (denaturation), 30 cycles at 56°C for 30 s (annealing), and 35 cycles at 72°C for 1 min (extension). An extra extension was carried out at 72°C for 7 min. The amplification products of primary PCR were again amplified with nested primers following same the protocol. The final amplification products/bands were analyzed on 1.5% agarose gel stained with ethidium bromide under ultraviolet (UV) light [Figure 1]. The PCR hence performed using Universal eubacterial 16S rDNA primers is referred to as the universal PCR in this research. Positive (DNA extracted from *Mtb* growth) and negative (double-distilled water) controls were run with each batch of samples analyzed. Documentation of gel was done by Gel doc system BIO-RAD made in the USA.

Polymerase chain reaction amplification of *hsp65*, *stkG*, or *fliC* gene

The samples positive for the bacterial DNA by universal PCR were further checked for the presence of *S. Typhi*,

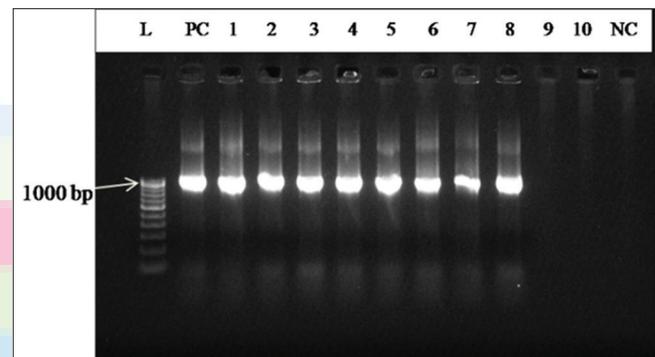


Figure 1: Gel image showing 1025bp size target amplicon after nested polymerase chain reaction using universal eubacterial 16S rRNA primers. L - Ladder; PC - positive control; 1-10 samples; NC: negative control

S. Paratyphi A, and or *Mtb* if any using second nested PCR reaction. This second PCR is referred to as the secondary PCR in this research. For a single sample, three sets of nested PCR were performed in 25 µl volume of reaction mixture. Each reaction mixture contained 10× reaction buffer (5 µl/sample), dNTPs (0.25 mM each), forward and reverse primers (10 pmole each targeting either *hsp65*, *stkG*, or *fliC* gene) and *Taq polymerase* enzyme (1 unit/reaction), and 1.5 mM MgCl₂. The template used was ranged between 50 ng and 100 ng of DNA extracted. Amplification was carried out on BIO-RAD system with a heated lid. The amplification condition was same as performed for universal PCR, except the annealing temperature and conditions which were as per Table 1. The final amplification products/bands were analyzed on 1.5% agarose gel stained with ethidium bromide under UV light [Figure 2]. Positive (DNA extracted from *Mtb* growth) and negative (double distilled water) controls were run with each batch of samples analyzed.

Documentation of gel was done by Gel doc system BIO-RAD made in the USA.

Statistical analysis

Fisher's exact test was used to compare categorical variables in different groups. A $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

All of the bone marrow samples were found to be culture negative at 7th day after aerobic incubation at 37°C. Out of a total 18 bone marrow samples tested through universal PCR, 16 (89%) samples could yield desired amplicon, while the remaining 2 (11%) samples were negative for it [Figure 1]. Table 2 summarizes the secondary PCR results of 16 samples detected positive through universal eubacterial 16S rDNA primers. The gel image of secondary PCR final amplified product shown in Figures 2-4 the desired amplicons were 3 (18.8%) for *Mtb*, 4 (25%) for *S. Typhi*, and 1 (6.2%) for *S. Paratyphi A* [Table 2]. The remaining 6 (50%) samples which could not be identified by secondary PCR were grouped under unidentified organism. A significant difference was noted between the detection of *S. Paratyphi A* and remaining unidentified organism ($P = 0.015$), the detection rates between other group remained insignificant.

Discussion

Blood and bone marrow is one of the largest organs in the body and is an important potential target organ for different kind of exposures to exogenous and endogenous factors.^[12] Culture failed to detect the presence of microorganisms in bone marrow samples proves the inferiority of culture over molecular test like nested PCR. The present work has shown that the 16S rDNA PCR can detect a wide range of organisms directly from clinical specimens. Thus, 16S rDNA PCR, if used in combination with routine culture techniques in the clinical microbiology laboratories will increase the diagnosis of bacterial infection from clinical specimens.

The two known pathogens detected were *Salmonella enteric* subspecies *enterica* serovars Typhi and Paratyphi in 31% of the cases in healthy individuals. This is really very surprising. However, when the diagnosis of acute typhoid fever becomes difficult, the bone marrow always yields the bacterium as the ratio between peripheral blood to bone marrow is 1: 36.^[13] This means *S. Typhi* and *S. Paratyphi* serotypes are normally present at higher concentrations in bone marrow than peripheral blood. It has been reported in cell culture experiments that serovar Typhi can replicate in human macrophages to reach an average of 14 organisms per cell.^[14] In contrast, the peripheral blood monocytes from

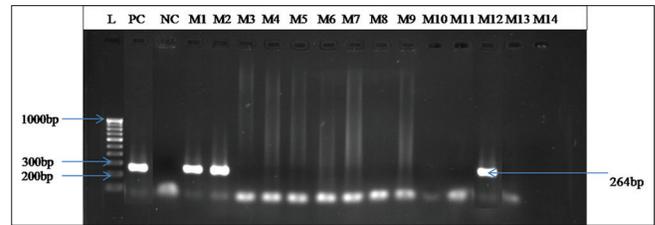


Figure 2: Gel picture of heat shock protein gene amplified product of *Mycobacterium tuberculosis* from bone marrow cases: L - Ladder (molecular marker 100 bp), PC - Positive control, NC - negative control, M1-M18 - bone marrow sample

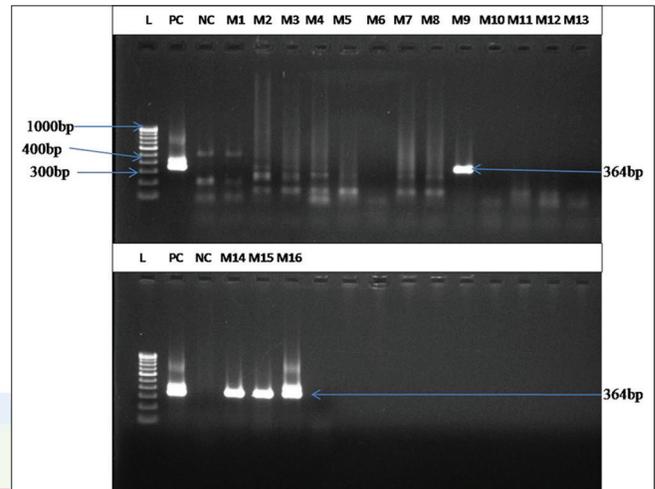


Figure 3: Gel image showing *flagellin* gene amplified product of *Salmonella Typhi* from bone marrow samples: L - Ladder (molecular marker 100 bp), PC - positive control, NC - negative control, M1-M18 - bone marrow sample. Product size is 364 bp

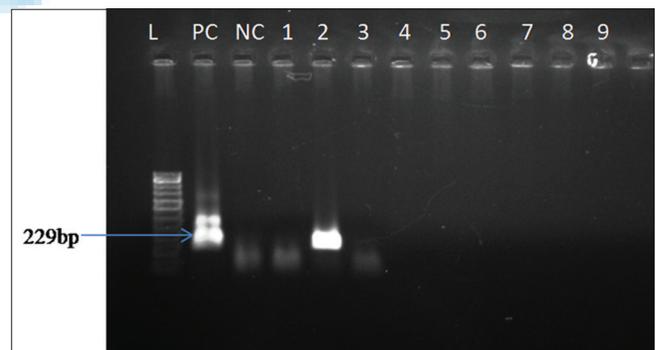


Figure 4: Gel picture of specific putative fimbrial (*stkG*) gene amplified product of *Salmonella Paratyphi A* in bone marrow samples L - Ladder, PC - positive control, NC - negative control, 1-18 - bone marrow sample, product size is 229 bp

patients infected with serovar Typhi contain an average of only 1.3 CFU/cell.^[15]

The exact location of serovar Typhi in human bone marrow is still to be explored. However, it has been suggested that there are at least three bacteria per cell in the bone marrow during human typhoid fever.^[13] As we know antibiotic therapy or the natural course of infection often fails to eradicate all the bacteria from the body in

Table 2: Secondary polymerase chain reaction results of 16 samples detected positive through universal eubacterial 16S rDNA primers

Microorganism	n (%)
Mtb	3 (18.8)
<i>Salmonella</i> Typhi	4 (25)
<i>Salmonella</i> Paratyphi A	1 (6.2)
Unidentified microorganism	6 (50)
Total	16 (100)

Mtb = *Mycobacterium tuberculosis*

some of the cases, this bacterium might take shelter in the bone marrow in healthy individuals.

The other known bacterium is *Mtb* which could be detected in 18.8% of the bone marrow. It is a facultative intracellular and evolved in such a way to protect itself in the macrophages. These findings imply that in the community healthy carriers are present, and sometimes due to alteration in immune response, they may result into full-fledged tuberculosis. Das *et al.* have stated that *Mtb* can persist in hostile intracellular microenvironments evading immune cells and drug treatment.^[2] However, the protective cellular niches where *Mtb* persists remain unclear. They have reported that *Mtb* may maintain long-term intracellular viability in a human bone marrow (BM)-derived CD271⁺/CD45⁻ mesenchymal stem cell (BM-MSC) population *in vitro*. They have also reported that *Mtb* resides in an equivalent population of BM-MSCs in a mouse model of dormant tuberculosis infection. Viable *Mtb* could be detected in CD271⁺/CD45⁻ BM-MSCs isolated from individuals who had successfully completed months of anti-*Mtb* drug treatment. These results suggest that CD271⁺ BM-MSCs may provide a long-term protective intracellular niche in the host, in which dormant *Mtb* can reside.

Through this technique, we could not identify 6 (50%) microorganism that detected positive by universal eubacterial 16S rDNA primers. This could be because in secondary PCR we targeted the common pathogenic bacteria only. The identification of these rare or uncommon microbes requires other molecular techniques such as plasmid DNA isolation and restriction digestion, followed by sequencing and blast analysis.

Conclusions

Based on the observations of the present study, it may be considered that bone marrow is not a sterile site as it was considered before. Even in asymptomatic people, different kind of bacteria including pathogenic bacteria such as *Mtb*, *Salmonella* spp., and many other microorganisms may be present as commensal. The main limitation of this study was small sample size. However,

further research involving a large number of cases might be able to give a definite answer.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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