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M. TUBERCULOSIS IN BONE MARROW AS COMMENSAL – A HIDDEN SOURCE FOR PRIMARY OSTEOARTICULAR TUBERCULOSIS !!

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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 by using eubacterial universal primers.

MATERIAL 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 PCR using Universal eubacterial 16S rDNA primers. The samples positive by universal PCR was further checked for the presence of S. Typhi, S. ParaTyphi, A and or *M. tuberculosis* 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%) for M. *Tuberculosis*, 4 (22%) for S.Typhi, and 1 (6%) for ParaTyphi A.

CONCLUSIONS: Even in asymptomatic cases other infectious agents like *M. tuberculosis, Salmonella spp.* and many other microorganisms may be present as commensal and may cause active clinical infection on certain conditions.

KEYWORDS : Bone marrow, PCR, S. Typhi, S. ParaTyphi, A, M. Tuberculosis

Introduction

The bone marrow which constitutes about 5% of total body weight in humans is pluripotent stem cells, with the ability to become a variety of cells i.e. red and white blood cells.[1] 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 may provide a niche for dormant *Mycobaterium tuberculosis*," mentions that the CD271+ stem cells are self-replicating, reside 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, a wide variety of organism have been isolated from the bone marrow of the patients who have had suffered from chronic diseases like leishmaniasis, brucellosis, etc. after they had been declared clinically cured, even in immunocompetent patients.

Our laboratory has carried out work related to screening for different bacteria in bone marrow of patients suffering from aplastic anemia and haematological malignancies (AML, CML, ALL, CLL etc.). We have observed the presence of *Salmonella species* and *Mycobacterium tuberculosis* in bone marrow collected from children suffering from aplastic anemia and also from cases of hematological malignancies (unpublished data). Further, we could detect *M. tuberculosis* in a patient suffering from chronic osteomyelitis also. Very high prevalence in 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 by using eubacterial universal primers. Further, to identify the spectrum of bacteria 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 Department of Orthopaedics 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. A total of 18 nonduplicate bone marrow samples of apparently healthy individuals were collected from patients admitted to Orthopaedics department having closed bone fractures. The bone marrow was drained aseptically during surgery from the cases that were planned for elective surgery.

Patient on medication causing bone marrow suppression, pregnancy, sero-positive for HBsAg, 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 15ml) from exposed bone marrow of patients with full aseptic precautions in operation theatre was sent to Department of Microbiology, IMS, BHU in a plane sterile vial. Out of this 10 ml of sample was inoculated in Brain Heart Infusion (BHI) broth and incubated at 37 ^{0}C. The broth was subsequently subcultured on blood agar at 7th days to look for any bacterial growth. The remaining volume of samples was stored at -20C temperature till further use.

MOLECULAR STUDY

Extraction of DNA:
DNA isolation was done using standard, phenol-chloroform method Sambrook *et al.*[4]

PCR 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).[5]

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 PCR; hspTBF2 and hspTBR2 were selected for amplification of a 218 bp nucleotide sequence for nested PCR (Table 1).[6]

Oligonucleotide primers for S. 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 96-118 and 522-501, respectively (Table 1). For nested PCR, oligonucleotide 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 C B *et al*.[7]

For the detection of S. Typhi, primers were used targeting flagellin (fliC) gene sequence as described by Song et al. and modified by Frankel (Table 1).[8,9] The first round PCR of both genes were amplified 495 and 537 bp whereas nested PCR amplified 364 and 377 bp respectively from amplified product of first round PCR**.**

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

PCR 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 mix contained 10 x reaction buffer (5 µl/sample), dNTPs (0.25mM each), forward and reverse primers (10 pmole each) and *Taq polymerase* enzyme (1 unit/reaction) and 1.5mM $MgCl_2$. The template used was ranged between 50ng-100ng 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 1min (denaturation), 30 cycles at 56 C for 30 s (annealing) and 35 cycles at 72 C for 1min (extension). An extra extension was carried out at 72 C for 7 min. The amplification products of primary PCR was 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 UV light (figure 1). The PCR hence performed using Universal eubacterial 16S rRNA primers is called referred to as the universal PCR in this research. Positive (DNA extracted from *M. tuberculosis* 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.

PCR Amplification of *hsp65, stkG,* or *fliC* gene
The samples positive for the bacterial DNA by universal PCR was further checked for the presence of S. Typhi, S. ParaTyphi, A and or *M. tuberculosis* 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 mix contained 10 x reaction buffer (5 µl/sample), dNTPs (0.25mM each), forward and reverse primers (10 pmole each targeting either *hsp65, stkG,* or *fliC* gene) and *Taq polymerase* enzyme (1 unit/reaction) and 1.5mM $MgCl_2$. The template used was ranged between 50ng-100ng 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 Table1. 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 *M. tuberculosis* 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.

Observation and Result
All of the bone marrow samples were found to be culture negative at 7^{th} day after aerobic incubation at 37^0C. 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). The secondary PCR of 16 samples, the desired amplicons were detected 3 (18%) for *M. Tuberculosis,* 4 (22%) for S.Typhi, and 1 (6%) for ParaTyphi A (Figure 2, 3 and 4).

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.[10] 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 broad-range 16S rDNA PCR can detect a wide range of organisms directly from clinical specimens. Thus 16S rDNA PCR, when used in combination with routine culture techniques in the clinical microbiology laboratories, increases the diagnosis of bacterial infection from clinical specimens.

The two known pathogens detected were *Salmonella enterica* subspecies *enterica* serotypes Typhi and Paratyphi in 28% of the cases in healthy subjects. 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.[11] 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**.**[12] In contrast, the peripheral blood monocytes from patients infected with serovar Typhi contain an average of only 1.3 CFU/cell.[13]

The exact location of serovar Typhi in human bone marrow has not been characterized. However, it has been suggested that there is a median of at most three bacteria per cell in the bone marrow during human typhoid fever. As we know antibiotic therapy or the natural course of infection often fails to eradicate all the bacteria from the body in some of the cases, this bacterium might take shelter in the bone marrow in healthy individuals.

The other known bacterium is *Mycobacterium tuberculosis* which could be detected in 17% 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 *Mycobacterium tuberculosis* (*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.

Conclusion

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 like *M. tuberculosis, Salmonella spp*. and many other microorganisms may be present as commensal. It may be potential source of active osteoarticular tuberculosis infection . However, more research involving a good number of cases might be able to give a definite answer.

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Table-1 Primers and amplification conditions targeting Mycobacterium tuberculosis complex, S. paratyphi A ,and S. Typhi

Target gene	Cycle	Primer names and sequences	Amplicon size	Annealing temperature & number of cycles	Reference
16S rDNA (Universal eubacterial primers)	Primary cycle	**16S F** 5′-TTG GAG AGT TTG ATC CTG GCT C-3′	1194 bp	56?C for 30 s for 30 cycles	N Okhravi, *et al.*, 2000 [5]
		16S R 5′-ACG TCA TCC CCA CCT TCC TC-3′			
	Nested cycles	**NF** 5′-GGC GGC AKG CCT AAY ACA TGC AAG T-3′	1025 bp	56?C for 30 s for 30 cycles	
		NR 5′-GAC GAC AGC CAT GCA SCA CCT GT-3′			
Heat Shock protein 65 (*hsp65or groEL2*)	Primary cycle	**hspTB*F1*** 5′ AAA AGC CGG ATG GCA ATT CG 3′	494 bp	65?C for 1 min 35 cycles	Rahaman H *et al.*, 2015 [6]
		hspTB*R1* 5′ ATT ACC GGC TTG GAC CCC CTG 3′			
	Nested cycles	**hspTB*F2*** 5′ ATG CGG CGC ACA CCG AAG ACAAG 3′	218 bp	65?C for 1 min 35 cycles	
		hspTB*R2* 5′ TCA ACG CGC TGT CTA GCT TGT C 3′			
Putative fimbrial gene *(stkG)*	Primary cycle	***stkG* F1 5′** CGTTTACTGAGGTCACAGGCATC-3′	427bp	51.5°C for 30 s for 30 cycles	Pratap C B *et al.*, 2014 [7]
		***stkG* R1 5′-**CACATTGTTCTCGGAGACCCCA-3′			
	Nested cycles	***stkG*F2 5′-**CAATGGCTTCTGGCGAACTGTC-3′	229bp	51.5°C for 30 s for 30 cycles	
		***stkG*R2 5′-**TGGAGAAAGATCAGACCACCGAG-3′			
Flagellin gene *(fliC)*	Primary cycle	**ST1 5′**-ACTGCTAAAACCACTACT-3′ **ST2 5′**-TTAACGCAGTAAAGAGAG-3′	495 bp	51.5°C for 30 s for 30 cycles	Frankel *et al.*, 1994 [9]
	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	

Figure 1: Gel image showing 1025bp size target amplicon after nested PCR using Universal eubacterial 16S rRNA primers. L Ladder; Pc Positive control; 1-10 samples; Nc Negative control

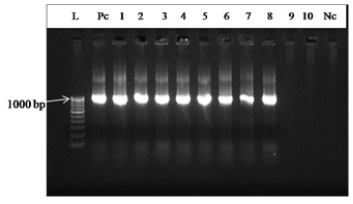

Figure 2: Gel-picture of heat shock protein (hsp) gene amplified product of Mycobacterium tuberculosis from bone marrow cases: L – ladder (molecular marker 100bp), PC – positive control, NC- negative control, M1- M18 – bone marrow sample.

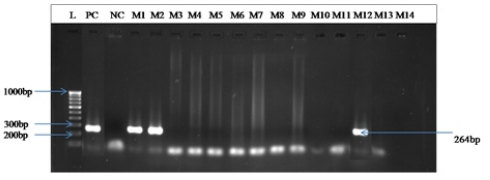

Figure 3: Gel image showing *flagellin* gene(fliC) amplified product of S. Typhi from bone marrow samples: L – ladder (molecular marker 100bp), PC – positive control, NC- negative control, M1- M18 – bone marrow sample. Product size is 364bp.

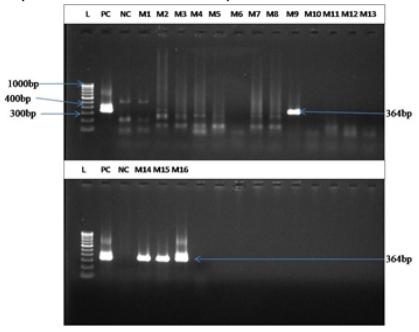

Figure 4: Gel picture of specific putative fimbrial (stkG) gene amplified product of *Salmonella* Paratyphi A in bone marrow samples L- Ladder, PC- Positive control, NC- Negative control, 1-18 :- bone marrow sample, product size is 229bp .