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*Full Length Research Paper*

# Conjugational transfer and survival of plasmid encoding silver and antibiotic resistance genes of *Acinetobacter baumannii* BL54, *E. coli* K12 J53.2 transconjugants and pseudomonas transformants in different soil microcosms

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**In this investigation we tried to transfer plasmid encoded silver and antibiotic resistance genes from *Acinetobacter baumannii* BL54 to *E. coli* k12 J53.2 and Pseudomonas (a soil microflora) by conjugation and transformation in different soil microcosms and study the survival of the isolated bacterium in each soil. Clay loam, fine clay, sandy and clay soils were collected from different area of mahrashtra in India. Microcosm was developed for each type of soil in the glass tube (150 x 25 mm) with 2 g soil moistened with 1 ml 0.5% sterile saline. The conjugation frequency was lowest in sterile clay soil with frequency of 0.2 x $10^{-6}$, while, it was maximum in clay loam soil with frequency of 0.6 x $10^{-6}$. Similarly, in non-sterile soil microcosms, the rate of conjugation was highest in clay loam soil with frequency of 0.09 x $10^{-6}$ while was lowest in sandy soil (0.03 x $10^{-6}$). Rapid death of the organisms was observed within 9 days of incubation in presence of selection in sandy soil, while in fine clay, survival of the organisms was extended beyond 11 days. The Pseudomonas transformant survived for more than 40 days in presence of selection. From above results it can be concluded that plasmid mediated silver and antibiotic resistant genes were transferred in different soil by conjugation process. However, the rate of conjugation was affected by soil type. Soil transformant considerably survived in fine clay containing high amount of organic carbon and neutral pH as compared to sandy soil.**

**Key words:** Conjugation, soil type, survival, plasmid, *Acinetobacter baumannii*.

## INTRODUCTION

Soil is a macrohabitat containing minute amounts of nutrients, and is subjected to temporal and spatial variation (Barkey et al., 1985). Plasmids have been found in many genera of soil bacteria (Chopade et al., 1985; Devanas et al., 1986) and play an important role in sur-vival of soil organisms in presence of metals or antibiotics in the soil environment. Conjugation in the soil showed that plasmid encoding gene for degradation of 3-chlorobezoate was transferred to indigenous soil flora (*Pseudomonas*) with

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**Table 1.** Chemical analysis of soil samples used in this study.

| Soil type | Source | pH | Electric conductivity Ohm $^{-1}$ /Cm $^{-1}$ | Organic carbon (%) | P (gm %) | K (gm %) | Organic matter (%) | Total soluble salt (%) | CaCO3 | Silt (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| Clay loam | Pune | 8.1 | 0.2 | 0.65 | 0.0012 | 0.007 | 0.72 | 0.8 | 0.9 | 30 |
| Fine clay (Black) | Pimperi | 7.5 | 0.2 | 0.79 | 0.0014 | 0.001 | 1.3 | 0.8 | 0.96 | 18.75 |
| Clay | Mahabale shwar | 6.3 | 0.12 | 0.549 | 0.008 | 0.001 | 0.94 | 0.6 | 0.88 | 16 |
| Sandy | mumbi beach | 8.6 | 0.15 | 0.04 | 0.001 | 0.0006 | 0.04 | - | - | 50 |

P= Phosphorous
K= Potassium
Soils were collected from 15cm depth above locations and subjected to analysis as described in text.
The above results are average of two independent experiments

high frequency (Hirkala and Germida, 2004). Talbot et al. (1980) reported transfer of antibiotic resistance plasmids among strains of *Klebsiella* in sandy soil. Population dynamics of $Hg^{2+}$ resistant bacteria showed that out of 76 isolates, 6 were capable to transfer this heavy metal resistance to the recipient cells in the soil (Naik et al., 1994; Pertsova et al., 1984). Appli-cation of $Cd^{2+}$ in laden sewage and grass land soil showed that $cd^{2+}$ resistant bacteria could not be able to compete with normal flora of the soil upon the long term exposure to cadmium (Poluektova et al., 2008). Devanas et al. (1986) suggested that conjugative plasmids may be the most important means of gene transfer in the soil as it was appeared a considerable decrease in number of some *E. coli* strains when recovered from sterile and non sterile soil microcosms as they lost the plasmids. Van Elsas et al. (1989) reported the survival of *P. fluorescence* containing plasmid RP4 and pRK501 and their stability in two soil microcosms. However, soil type profoundly effected the host survival. Indeed the indigenous microbiota of the soil may directly affect the conjugation process in the soil (Shakibaie et al., 1999, 1998). Field and soil microcosm studies on the survival and conjugation of a *P. putida* strain bearing a recombinant plasmid, pADPTel (Pertsova et al., 19847).

Majority of studies on transfer of the plasmids in soil microcosms have been conducted in Enterobacteriaceae (Krasovsky and Stotzky, 1987) and there is a paucity of information exist about conjugation and survival of plasmid mediated antibiotic and metal resistance bacteria in the soil. In previous study (Shakibaie et al., 1999, 1998, 2003) we isolated a species of *Acinetobacter baumannii* encoding conjugative plasmid responsible for silver and antibiotic resistance genes. In present investigation we tried to transfer this plasmid by conjugation to *E. coli* k12 J53.2 and by transformation to *Pseudomonas* (a soil microflora) in different sterile and non sterile soil microcosms and study survival of these plasmids encoding bacteria and Pseudomonas transformant in different soil microcosms.

## MATERIALS AND METHODS

### Collection of soil samples and developing soil microcosm

Clay loam, fine clay (forest, black), sandy and clay (red, latertic) soils were collected around different area of mahrashtra provenience in India. Thirty gram of each soil was removed by spade from 15 cm depth and collected in polythene bag. The soils were dried over night at 30°C and subjected to grinding with pestle followed by sieving through 0.242 mm pore size sieve. The processed soils were subjected to physicochemical analysis as shown in Table 1. Organic carbon was determined by Walkey Black potassium dichromate oxidation method and available phosphorus was determined by Olson method (Redford et al., 1981). The soils were then divided in two parts and one was sterilized by autoclaving at 121°C for one hour. Microcosm was developed for each type of soil in the glass tube (150 x 25 mm) with 2 g soil moistened with 1 ml 0.5% sterile normal saline and used for gene transfer and survival studies.

### Conjugation in soil

Conjugation was carried out in each sterile and nonsterile soil as described by Naik et al. (1994). Briefly, 1 ml O/N growth of *A. baumannii* ($10^7$/ ml) in Luria -Bertani medium as donor and 1 ml of *E. coli* K12 J53.2 (Rif $^r$) as recipient were added to each soil microcosm and incubated for 24 h at 30°C. With help of a sterile glass rod approximately 20 mg of soil was withdrawn and suspended in sterile saline and serially diluted. 0.2 ml aliquot of each dilution was spread in to Lactose electrolyte deficient medium (CLED) agar selective for transconjugant (Ag 200 µg/ml + Te 100 µg/ml + ampicillin 200 µg/ml and Rif 100 µg/ml). The frequency of conjugation was then calculated as number of colonies grown in this medium divided by number of recipient cells.

### Transformation and plasmid stability

Transformation and stability of plasmid were determined by shakibaie et al. (1998) as previous described. Briefly, Plasmid DNA was prepared with the Qiagen plasmid purification kit (Qiagen, Hilden, Germany). Overnight culture of *A. baumannii* BL54 were grown in LB broth with 100 µg AgNO3, washed twice in 0.9% NaCl and adjusted to a titer of $1\times10^9$ $ml^{-1}$. To 20 µl of this suspension 10 µl of plasmid DNA in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and 50 mM ice cold $CaCl_2$ was added and the mixture was spotted onto LB

**Table 2a.** Conjugation between *A. baumannii* and *E. coli* K12 J53.2 in different sterile soil microcosms.

| Soil type | *A. baumannii* (CFU/g soil) | *E. coli* K12 J53.2 (CFU/g soil) | Frequency of conjugation | Marker co-transferred |
|---|---|---|---|---|
| Fine clay | $1.6 \times 10^7$ | $2.2 \times 10^7$ | $0.3 \times 10^{-6}$ | Te, Ag, Amp |
| Sandy | $2 \times 10^7$ | $1.2 \times 10^7$ | $0.3 \times 10^{-6}$ | Ag, Te, Amp |
| Clay loam | $3 \times 10^7$ | $2.2 \times 10^7$ | $0.6 \times 10^{-6}$ | Amp, Te, Ag |
| Clay | $1.2 \times 10^7$ | $3 \times 10^7$ | $0.32 \times 10^{-6}$ | Te, Ag, Amp |

**Table 2b.** Conjugation between *A. baumannii* and *E. coli* K12 J53.2 in different non sterile soil microcosms.

| Soil type | *A. baumannii* CFU/g soil | *E. coli* K12 J53.2 (CFU/g soil) | Frequency of conjugation | Marker co-transferred |
|---|---|---|---|---|
| Fine clay | $1.2 \times 10^7$ | $3.1 \times 10^7$ | $0.07 \times 10^{-6}$ | Te, Ag, Amp |
| Sandy | $2.1 \times 10^7$ | $2 \times 10^7$ | $0.03 \times 10^{-6}$ | Ag, Te, Amp |
| Clay loam | $6.2 \times 10^7$ | $1.6 \times 10^7$ | $0.09 \times 10^{-6}$ | Amp, Te, Ag |
| Clay | $2 \times 10^7$ | $3.8 \times 10^7$ | $0.07 \times 10^{-6}$ | Te, Ag, Amp |

A control of conjugation was carried out to check presence of spontaneous mutation. It was below limits of detection.

agar. Incubation was for 20 h at 37°C. Appropriate dilutions of the resuspended cells were plated on LB agar containing 100 µg $AgNO_3$ + 100 µg Rif (titer of transformants) and Rifampicin (titer for recipients). Colonies were counted following incubation for 48 h at 37°C.

**Survival of *A. baumannii* BL54, *E. coli* K12 transconjugant and Pseudomonas transformant in different soil microcosms**

2 ml of over night growth of each isolate ($10^8$ CFU/ml) was inoculated separately in to 4 g of sterile and nonsterile (Fine clay, clay loam, clay and sandy) soil microcosms. All tubes were incubated up to 44 days. At two days interval 5 mg soil was taken from each soil microcosm and suspended in 10 ml 0.85% sterile saline and serially diluted. 0.1ml of each dilution was spread into CLED agar medium containing 25 µg/ml Tetracycline and 200 µg/ml $AgNO_3$ and into CLED agar. After 24 h of incubation at 30°C, total viable count of bacteria in each soil then was calculated.

## RESULTS

Chemical composition of different soil microcosms used in this study is shown in Table 1. Table 1 indicates, the forest soil contain considerable amount of organic matter as well as phosphorous and neutral pH while, sandy soil contain minimum amount of nutrient as well as alkaline pH (8.6). The results of conjugation in sterile and nonsterile soil microcosms are shown in Table 2a, b. The conjugation frequency was lowest in sterile Clay soil with frequency of $0.2 \times 10^{-6}$, while, it was maximum in clay loam soil with frequency of $0.6 \times 10^{-6}$. Similarly, in non sterile soil microcosms (Table 2b), the rate of conjugation was highest in clay loam soil with frequency of $0.09 \times 10^{-6}$ while was lowest in sandy soil ($0.03 \times 10^{-6}$). This indicate that transfer of metal and antibiotic resistance genes indeed occur in different soil microcosms and soil type influenced the frequency of conjugation. Concurrent testing of transconjugant in presence of ampicillin 50 µg/ml + Rif 100 µg/ml, Te 50 µg/ml + Rif and Ag 200 µg/ml + Rif showed simultaneous transfer of these genes to the recipient cells. Transfer of plasmid from *A. baumannii* to *Pseudomonas* a soil microflora had occurred by transformation process (data not shown) with frequency of $0.4 \times 10^{-6}$. Co-transfer study of transformant indicated simultaneous transfer of above antibiotic resistant markers to *Pseudomonas*.

The survival of *A. baumannii, E. coli* K12 J53.2 transconjugant and Pseudomonas transformant was studied in different types of soil as shown in Figures 1 and 2. Rapid death of the organisms was observed within 9 days of incubation in presence of selection in sandy soil, while in fine clay soil survival of the organisms was extended beyond 11 days. This decrease in plasmid bearing cells was apparently the result of partitioning of the plasmid without duplication in daughter cells. The above conclusion was supported from comparatively high CFU of the organisms at the start of experiment as shown in Figure 1. This indicated that expression of resistant genes on plasmid was inhibited after long incubation under starvation condition. The above results were further supported by study of the viable count of pseudomonas transformant in presence and absence of selection as shown in Figure 2. The organism survived for more than 44 days in the absence of selection and for40 days in presence of selection. This indicates that expression of these resistant genes was also depending on physiology of the organism as well. Since *Pseudomonas* is as soil

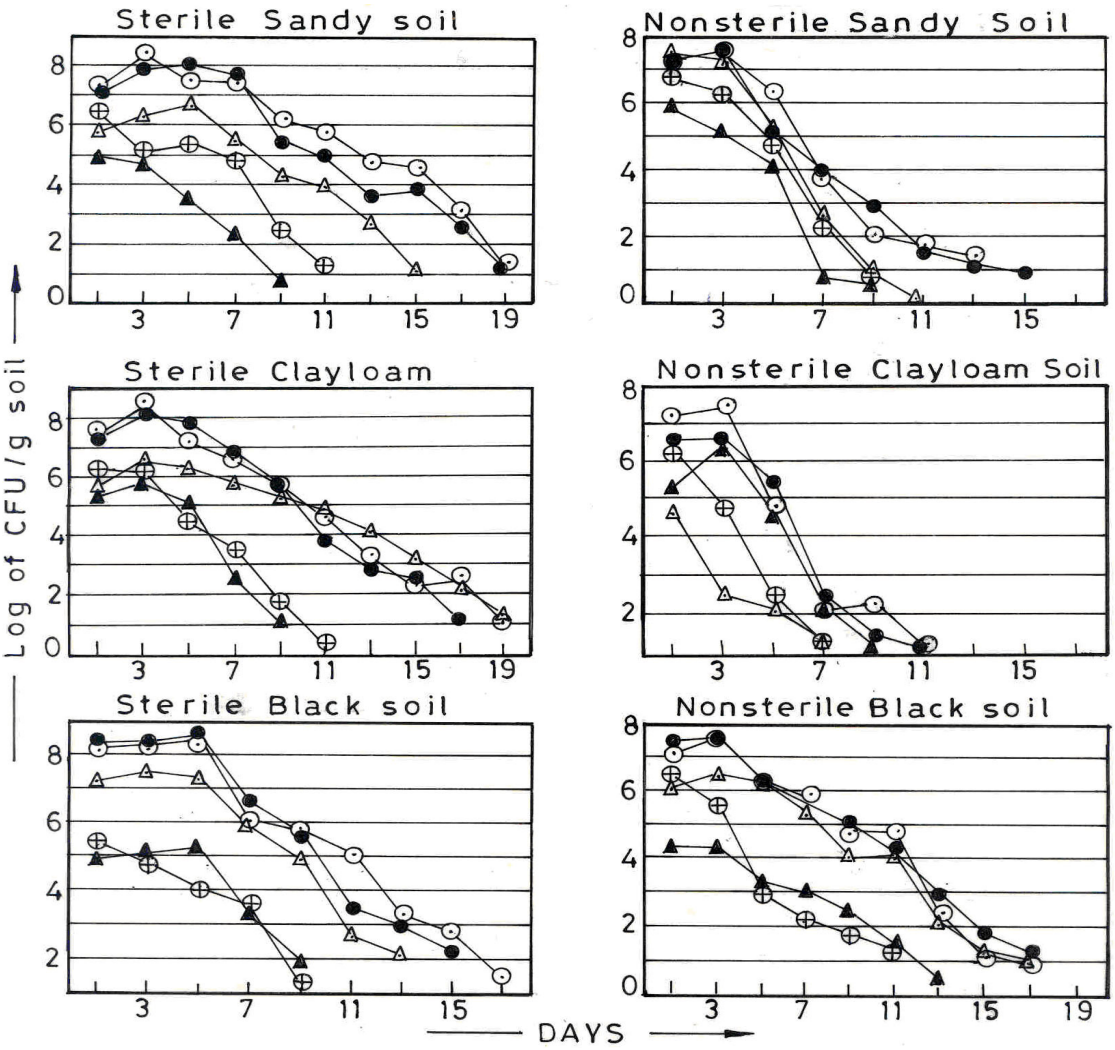


**Figure 1.** Survival of *A. baumannii* and *E. coli transconjugant* in sterile and nonsterile sandy, clay, clay loam and fine clay (forest) soil microcosms.
⊙ ; *A. baumannii* plated on CLED agar, ●; *E. coli* plated on CLED agar, □; *A. baumannii* plated on CLED agar + 200µg/ml $AgNO_3$ ,▲; *E. coli* transconjugant plated on CLED agar + 200µg/ml $AgNO_3$, ▲; *E. coli* transconjugant plated on CLED agar + 50µg/ml Te.

isolate it better adapted in starvation condition, while, *A. baumannii* and *E. coli* transconjugant being zymogenous were not able to survive in starvation under pressure of antibiotic and metal containing medium.

## DISCCUSSION

The major concern about introduction of plasmid containing organisms in soil is not only their potential adverse ecological impacts on the homeostasis of soil but also on the bioremediation of pollutants like heavy metals from different type of soil. Krasovky and Stotzky, (1987) demonstrated that indigenous microflora of soil directly effects the survival of plasmid bearing bacteria in nonsterile soil.

In our study plasmid encoding Acinetobacter was quite stable in all soil tested. The stability was not depending on type of soil but it was depend on type of host. Chemical composition of different soil microcosms used in this study indicates that, the forest soil contain considerable amount of organic matter as well as phosphorous and neutral pH (7.5) while, sandy soil contain minimum amount of nutrient as well as alkaline pH (8.6). Therefore, it is appropriate to suggest that genetic markers could not be expressed up on long term in starvation condition in sandy soil. This was supported by study of population dynamics of Acinetobacter, *E coli* transconjugant and Pseudomonas transformant in different types of soil microcosms. This also indicated that plasmid have no role in long term survival of the organisms in soil. Infact, viable count declined rapidly when cells were plated in

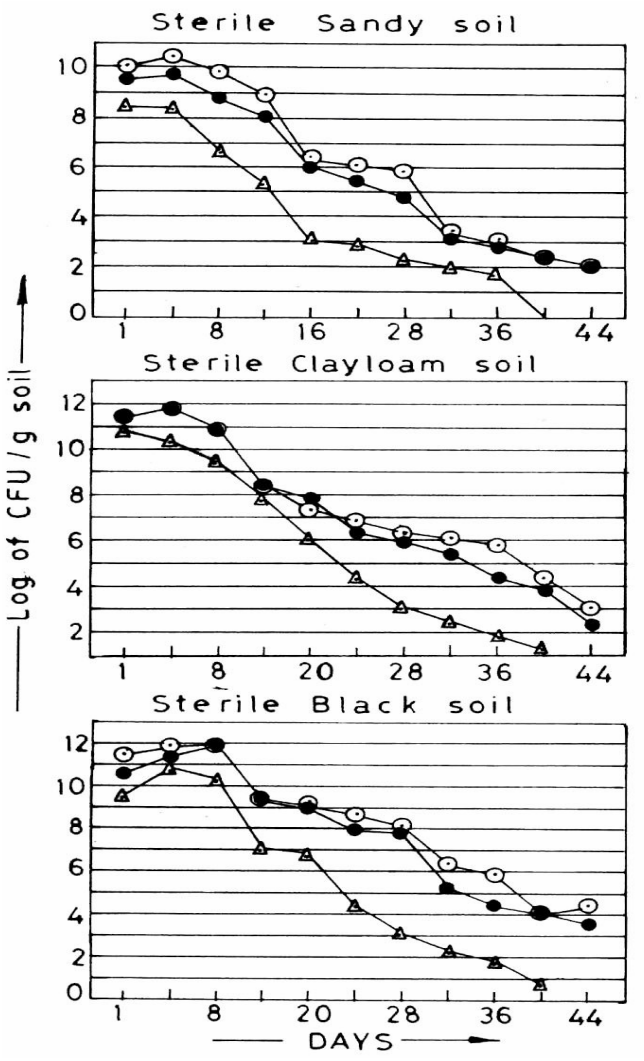


**Figure 2.** Survival of Pseudomonas transformant (an indigenous soil microflora) in sandy, clay loam and fine clay (forest) soil microcosm

⊙; Organism plated on CLED agar, ●; Organism plated on CLED agar + 50 µg/ml Te, ▲; Organism plated on CLED agar + 200µg/ml $AgNO_3$

metal and antibiotic containing medium when plasmid was present in the cells. The viable count decreased rapidly in sandy soil, while in forest soil it was prolonged by few days.

Endogenous energy source is an important factor in microbial survival and it may be that autochthonous bacteria have a mechanism for reducing metabolic rate in order to enhance their survival under starvation condition (Trevors, and Oddie, 1986). Shakibaie et al. (1999) reported that accumulation of silver is an energy dependent process. It is quite possible nutrients were not available in soil and affect long term survival of the organisms. At present, research is carrying out by our group in this regard. Recently, horizontal transfer of antibiotic resistant genes was studied in sewage and lake water by Shakibaie et al. (2009). It was observed that the rate of conjugation was two fold high in sewage than in lake water. The physico-chemical parameters of water also were contributed to gene transfer by conjugation.

## Conclusion

From above results it can be concluded that metal and antibiotic resistant genes were transferred in different soil types by conjugation process. However, the rate of conjugation was depending on soil type as well as type of host. The plasmid containing soil microbiota transformant considerably survived under selective and non selective conditions and soil type also play role in the survival of isolated organisms as well.

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