Original Article

Assessing Survival of Transgenic Bacteria, *Serratia* AS1 and *Enterobacter cloacae*, in Sugar Bait, White Saxaul Plant (*Haloxylon persicum*) and Rodent Barrow's Soil, A Contained-Field Study for Paratransgenesis Approach

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Abstract

Background: The viability and persistence of engineered bacterium candidates in field conditions is one of the considerable challenges in the paratransgenesis approach to fighting vector-borne diseases.

Methods: In this study two engineered bacterium candidates to produce paratransgenic sand flies, *Serratia* AS1 and *Enterobacter cloacae* expressing m-Cherry fluorescent were applied on the leaves of the white saxaul plant (*Haloxylon persicum*), sugar bait, and rodent burrow soil and their persistent time was tested in desert condition, Matin Abad County, Isfahan, August 2022. A PBS suspension of 10^9 cells/ml was used for sugar bait, spraying on plant leaves (~10 cm^2) and 10 cm^2 of rodent burrow soil. Sand fly samples were taken daily and were plated on LB Agar and the fluorescent cells were counted after 24 hours.

Results: Time course in general caused a decrease in the number of bacteria for both strains. The two strains were persistent in sugar bait and on plant leaves for four days and on soil for two days. Although there were slight differences between the number of the bacteria in sugar baits, which was not significant ($P< 0.05$). The number of *E. cloacae* surviving on plant and in soil were significantly ($P< 0.0001$ and $P= 0.046$) higher than *Serratia* AS1.

Conclusion: This study shows that plants or sugar bait are useful routes for delivery of the transformed bacteria for the paratransgenesis approach, although, the bacteria ought to be sprayed on plants or sugar baits should be replaced with new ones in four days intervals.

Keywords: Paratransgenesis; Symbionts; Sand fly; Leishmaniasis; Vector-borne diseases

Introduction

Paratransgenesis involves genetic manipulation of some suitable microbiota of an arthropod vector, such as a mosquito or sand fly. The goal of paratransgenesis is to disrupt the pathogen's life cycle and lessen its capacity to spread by using genetically altered microbes that can be delivered into the gut or other tissues of the vector (1).

The prospective benefits of using paratransgenesis are considerable, it is a very promising approach for controlling the spread of parasitic diseases, such as leishmaniasis. It offers a more targeted method compared to traditional control such as insecticides that kill insects regardless of whether they are useful or not. However, research on paratransgenesis against

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parasitic diseases such as leishmaniasis is in its early stages, and there are some challenges, such as finding an effective delivery system for introducing the transgenic microorganisms to vectors, which should be solved before this approach can be used as a practical control strategy (2–4).

Zoonotic cutaneous Leishmaniosis (ZCL) caused by *Leishmania major* is a widespread zoonotic disease that can be transmitted from animals' reservoirs to humans by blood-sucking phlebotomine sand flies such as *Phlebotomus papatasi* (5–9).

Rodents of the subfamily Gerbillinae, including the great gerbils *Rhombomys opimus,* are the primary reservoir hosts of ZCL and are the most dominant mammals recorded from the deserts in Old World (7,10,11). Rodent burrows are the most important shelter and breeding sites for Phlebtominae sand flies (12). The main diet of rodents, including great gerbils is herbivory and depends on metabolic water present in succulent plants. In central Iran, *Haloxylon* spp. and *Salsola* spp. plants are found dominantly, being stored in rodent complex tunnel systems (12), and or consumed by rodents. Consequently, the bacteria on the plants will be consumed by rodents and transiently pass through the rodent alimentary tract and are delivered to larval habitats with rodent feces (13). Also, the infected plant materials stored in depth of rodent canals in turn could infect sand fly larval habitats. The feces or other infected rotten organic materials will be ingested by sand fly larvae and may be transstadially transmitted to the adult stage of sand flies (14, 15). Therefore, the *Haloxylon* plants can be considered as a target site to deliver transformed bacteria in the field.

Sugar meals are essential for the migration and development of *Leishmania* parasites within sand fly gut. In addition, sugar meals are important for the sand fly survival, particularly throughout blood digestion, probably supporting the insect energy requirements. Sand flies normally obtain sugar meals in nature through feeding via a sugar source such as plant sap between blood meals (12, 14–17) or other sources such as honeydew of aphids (18). The sand fly sugar feeding behavior leads to develop Attractive Toxic Sugar Baits (ATSB) to control sand flies (19–23).

Soils around the rodent burrows also can serve as a site for the delivery of the transformed bacteria for the paratransgenesis approach. Rodents' skin (legs, tail, and abdomen skin) can be infected by bacteria while the animals move in and out of the burrow. The infected skin can consequently infect sand fly larval breeding places in rodent canals or directly infect sand fly females while they are probing and taking a blood meal on the animal body (24).

Our previous laboratory and field studies revealed that two genetically engineered bacteria, *Serratia* AS1, and *Enterobacter cloacae*, are suitable candidates for paratransgenic control of leishmaniasis (14, 24–27). These bacteria were found in sand flies (12, 28–31) and genetically manipulated to produce effector molecules that interfered with *Leishmania* development in the sand fly gut and consequently reduced the chance of parasite transmission (13, 14, 25, 32). Also, these two bacteria were used successfully in the paratransgenesis strategy for combating malaria in laboratory conditions (15, 26, 33, 34).

One of the faced challenges in introducing transgenic bacteria to insect vectors is that the bacteria should survive in the surrounding environment, so the vector comes in contact and will be infected with the transformed bacteria. In this study, we evaluate the time course on stability and persistence of *Serratia* AS1 and *E. cloacae* expressing an m-Cherry fluorescent protein that was applied on white saxaul plant leaves, sugar bait, and rodent burrow soil in the harsh desert condition of Matin Abad County, Esfahan.

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Materials and Methods

The study area for the vivarium setup

This study was conducted in Matin Abad County, Esfahan in central Iran with the following geographical coordinates: 33°44'59.3"N 51°59'59.9"E with an altitude of 981 meters above sea level (Fig. 1). The study area falls under the hot desert climate (BWh) in Köppen climate classification and (BW) in Trewartha climate classification, with cold winters and hot, sometimes very hot summers climate, where annual rainfall is about 108 mm, and the relative humidity (RH) varies between 15 % and 51%. At the time of experiments (22nd July till 22nd August 2022), the minimum and maximum daily temperature ranges were 25–31 ℃ and 38–51 ℃ respectively and RH ranged from 10–18%. This site was located on the border of the sand dunes and vast semiarid grass-covered plain and is one of the ZCL endemic foci in Iran (35). The selected area meets the desired criteria which consists of *Ph. papatasi* as the proven vector of ZCL, *R. opimus* as the main animal reservoir host, and the plants (*Haloxylon persicum*) that are being consumed by both the vector and reservoir host animals.

Vivarium set up

The contained-field set-up (Vivarium) was built using a scaffold framework measuring 15*6*2.5 m (Fig. 2A). The framework was fully covered with a finely meshed sand fly net (Fig. 2B) and protected by green plastic shade all around the enclosure against wild animals (Fig. 2C). The interior of the sand fly net was divided into two equal sections and were accessible by zipper doors, each chamber was used for one of the bacteria (Fig. 2D).

Each side of the net contained at least 1) five shrubs of *H. persicum*, the dominant vegetation in the area and the nectar source for sand flies and food for the rodents; 2) three apparent separate active *R. opimus* colonies (each one had 5–7 entrance ways and were far from each other by at least three meters), and 3) a natural population of wild *Ph. papatasi*.

Establishment of transgenic bacteria expressing mCherry protein

Serratia AS1 is a Gram-negative, facultative aerobic, rod-shaped bacterium that belongs to the Enterobacteriaceae family. This bacterium is a member of the insect microbiome and can be found in different arthropods (12, 36–39). *Serratia* also was isolated from a laboratory strain of *Ph. papatasi* in the sand fly insectarium, School of Public Health, Tehran University of Medical Sciences. A modified *Serratia* strain named *Serratia* AS1-mCherry was gifted from Prof. Jacobs-Lorena from the Department of Molecular Microbiology and Immunology, Malaria Research Institute, Johns Hopkins Bloomberg School of Public Health. It was created by integrating a mCherry fluorescent protein gene-carrying pBAM2 plasmid. The plasmid was constructed by substituting the beta-lactamase (ApR) gene of plasmid pBAM1 with an expression cassette (15, 40). *Enterobacter cloacae* was transformed with pBAM2-mCherry plasmid using the heat shock method. *Enterobacter cloacae* subsp. *dissolvens*, a rod-shaped, gram-negative bacterium belonging to the family Enterobacteriaceae, was isolated from the microflora of *Ph. papatasi* from a ZCL area in Isfahan, central Iran (12). A single colony of mCherry-fluorescent bacteria was extracted and cultured on Luria-Bertani (LB) agar at 28 °C and 37 °C for *Serratia* AS1 and *E. cloacae*, respectively. Bacterial stocks were prepared and stored in –80 °C for further use.

Bacterial suspension preparation

The transgenic bacteria were cultured in LB broth in a shaking incubator at 28 °C or 37 °C based on the bacterial strain. Once the OD_{600} reached 1 (approximately 10^9 cells per ml), the cells were centrifuged (3,000 g, 10 min), washed twice using sterile phosphate-buffered saline (PBS), and resuspended in 10%

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(wt/vol) sterile sucrose solution or 1x sterile PBS (depends on the experiments) to obtain 10^9 cells/ml suspension.

Stability of transgenic bacteria on sugar bait

To examine the stability of the bacteria in sugar bait in contained-field conditions, a 100 ml suspension containing 10^9 cells per ml of the transformed bacterial was poured into a 120 ml container and equipped with sterile cotton pads or paper towels, a wick was made so that the sugar solution was accessible to sand flies (Fig. 3A).

To prevent the ants from attacking the sugar bait, the sugar bait containers were hung from the top of the metal scaffold by a rope so that they were 5–10 cm far from the ground. The sugar baits were installed at four square meters along the length of the structure and placed at two meters from the wall of the vivarium (Fig. 2D) at sunset time. Due to the hot weather and strong evaporation of liquids, 30–50 ml ddH_2O was added to the sugar baits daily (at sunset) so that they didn't dry out. To perform the stability test, samples were taken daily from the container at sunset. For taking the samples, the existing wick was detached, and then 100 microliters of the solution were removed using a pipette. The removed solution was used to prepare a solid agar culture containing the apramycin (80 µg/ml) antibiotic, and the number of colonies (Colony Forming Units: CFUs) of the desired fluorescent bacteria were counted the next day. Serial dilution technique (https://microbeonline.com/serial-dilution-method/) was used if the number of cells per petri dish was more than 300. The transformed and the wild-type bacteria were used as positive and negative controls respectively.

Stability of the transgenic bacteria on plants

The purpose of this experiment was to determine if the bacteria sprayed on the plants were stable enough to be picked up by sand flies. To do so, the bacteria were cultured in LB Broth medium and after reaching the desired density ($OD6_{00}$ = 1 which has 10^9 bacteria per ml) were centrifuged. After washing twice with PBS, the pellet was resuspended in 10% sugar solution and was sprayed on the plant leaves (Fig. 3B). To perform this step, three plants were selected for each bacterium in each part of the vivarium, and 50 ml of the bacterial suspension were sprayed on each plant, in which all the aerial parts of the plant became wet and received the bacterial suspension. Approximately 10^9 cells were used per 10 cm^2 of the plant leaf. The experiment was performed in three replicates. To perform the stability test, samples were taken from the plant daily; 0.1 g of the plant leaf was cut off with sterile scissors and placed in a microtube containing 0.9 ml of liquid culture medium; and immediately, using the serial dilution technique, the liquid culture medium was added on a solid culture medium (LB Agar) containing the apramycin (80 µg/ml) antibiotic and incubated at 28 °C or 37 °C based on the bacterial strain. The number of fluorescent colonies of bacteria (CFUs) was counted after 24 hours. It should be noted that sampling was done at sunset.

Stability of transgenic bacteria in rodent's burrow soil

The purpose of this experiment was to determine if the bacteria were stable enough in the entrance of *R. opimus*'s burrow to encounter the rodent body. The infected animal can then transfer the bacteria to the nest and consequently infect the materials in the nest or the bacteria can be picked up by adult sand flies during blood meals in the nest. For this purpose, like the previous test, a liquid culture of the bacteria was prepared, and after centrifugation and washing bacteria with PBS, they were re-suspended in 1xPBS. 25 ml of the suspension was sprayed at each entrance and surrounding the rodent's burrow (about 10^9 cells per ml per 10 cm^2) (Fig. 3C). The soil samples were taken daily. The removed soil

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samples were cultured at 28 ℃ or 37 ℃ on LB agar medium containing the apramycin (80 µg/ml) antibiotic using serial dilution technique, and the transgenic bacterial numbers (CFUs) on the medium were counted after 24 hours.

Statistics analysis

The significance of bacteria's relative abundance in various samples was investigated using the Mann-Whitney U test. The student's t-test was performed to compare Means. SPSS version 27 (2020) was used for all analyses. At $P< 0.05$, statistics were deemed significant.

Results

Survival of the transgenic bacteria on sugar bait

The *Enterobacter cloacae* and *Serratia* AS1 expressing mCherry protein were viable on sugar baits for up to four days (Table 1). The bacterial populations were significantly decreased over time ($P< 0.0001$). Statistical analysis (Two-Way ANOVA) showed that the differences in the number of bacteria between days were significant for both bacteria ($P< 0.0001$) and the CFUs reached zero on the fifth day. However, the differences in the number of cells between the two bacteria were not significant ($P> 0.05$) and showed a similar trend (Fig. 4).

Survival of transgenic bacteria on plants

The results of the stability experiment of the bacteria are shown in Tables 2 and Fig. 5. The stability of both *E. cloacae* and *Serratia* AS1 bacteria on plant leaves was maximum of 4 days. Statistical analyses (ANOVA analysis) showed that the differences in the number of bacteria between days are significant for both bacteria ($P< 0.0001$) and the number of bacteria decreased significantly over the course of time and reached zero on the fifth-day post application. Also, the differences in the number of bacteria between the two strains are significant ($P< 0.0001$) and the stability of *E. cloacae* bacteria was significantly higher than *Serratia* AS1 during different days.

Survival of transgenic bacteria in soil

The stability of both bacteria on the soil of the rodent burrow was very low in the contained-field conditions and took no longer than two days (Table 3 and Fig. 6).

Table 1. The stability of *Enterobacter cloacae* and *Serratia* AS1 mCherry bacteria on sugar bait in the contained field (desert) condition of Matin Abad, Badroud, Esfahan Province, central Iran, 2022

Sampling Time (Day)	*Enterobacter cloacae*-mCherry			*Serratia* AS1-mCherry		
	1st repeat (CFU)	2nd repeat (CFU)	3rd repeat (CFU)	1st repeat (CFU)	2nd repeat (CFU)	3rd repeat (CFU)
1	112×10^6	117×10^6	128×10^6	106×10^6	115×10^6	142×10^6
2	62×10^6	51×10^6	28×10^6	32×10^6	45×10^6	53×10^6
3	9×10^6	8×10^6	11×10^6	4×10^6	5×10^6	7×10^6
4	1×10^6	0	0	0	1×10^6	0
5	0	0	0	0	0	0

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Table 2. The stability of *Enterobacter cloacae* and *Serratia* AS1 bacteria on plant leaves of *Haloxylon persicum* in the contained field (desert) condition of Matin Abad, Badroud, Esfahan Province, central Iran, 2022

Sampling Time (Day)	*Enterobacter cloacae*-mCherry			*Serratia* AS1-mCherry		
	1st repeat (CFU)	2nd repeat (CFU)	3rd repeat (CFU)	1st repeat (CFU)	2nd repeat (CFU)	3rd repeat (CFU)
1	72×10^6	77×10^6	86×10^6	63×10^6	66×10^6	59×10^6
2	53×10^6	44×10^6	31×10^6	22×10^6	31×10^6	33×10^6
3	25×10^6	28×10^6	12×10^6	8×10^6	13×10^6	15×10^6
4	8×10^6	0	4×10^6	0	2×10^6	4×10^6
5	0	0	0	0	0	0

Table 3. The stability of *Enterobacter cloacae* and *Serratia* AS1 bacteria on the soil of rodent burrow in the contained field (desert) condition, Matin Abad, Badroud, Esfahan Province, central Iran, 2022

Sampling Time (Day)	*Enterobacter cloacae*-mCherry			*Serratia* AS1-mCherry		
	1st repeat (CFU)	2nd repeat (CFU)	3rd repeat (CFU)	1st repeat (CFU)	2nd repeat (CFU)	3rd repeat (CFU)
1	66×10^6	83×10^6	72×10^6	54×10^6	69×10^6	28×10^6
2	11×10^6	16×10^6	4×10^6	8×10^6	2×10^6	0
3	0	0	0	0	0	0

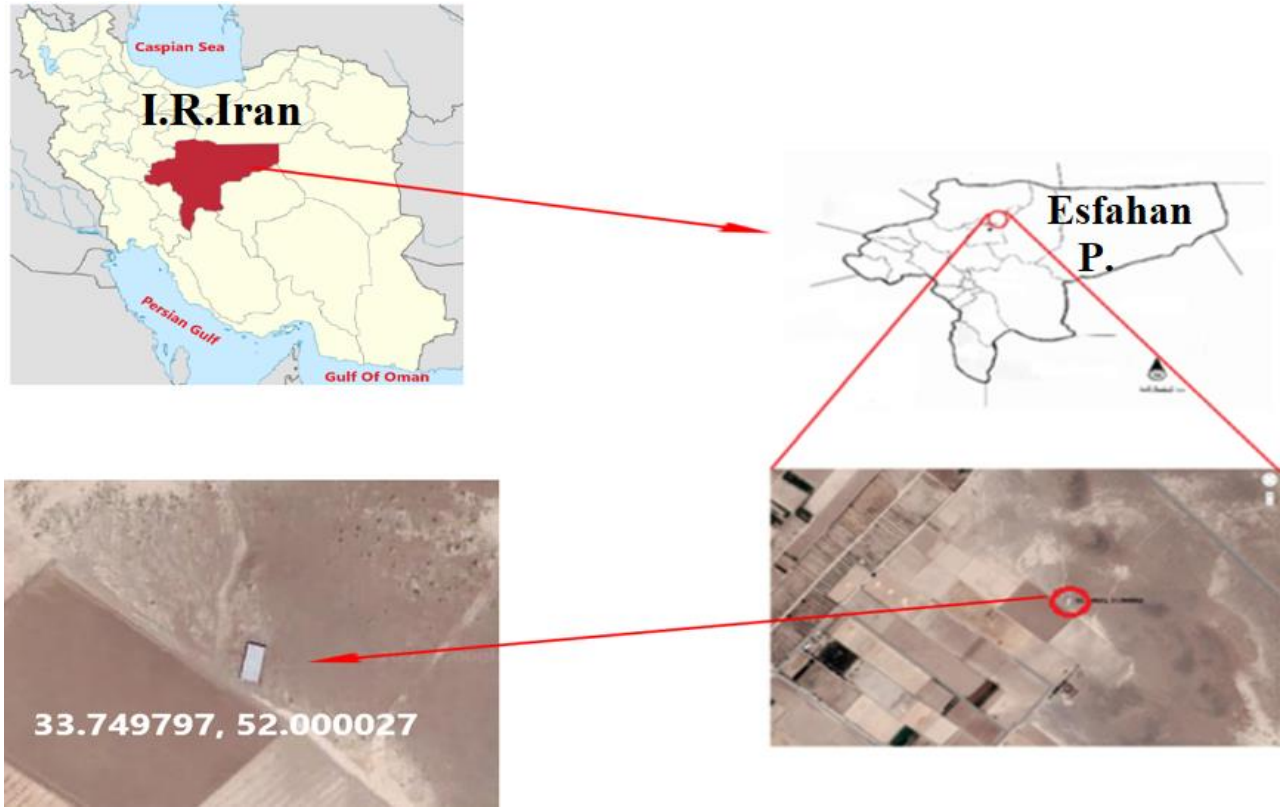

Fig. 1. Location of the contained-field site used in this study, Matin Abad, Badroud, Esfahan Province, central Iran, 2022

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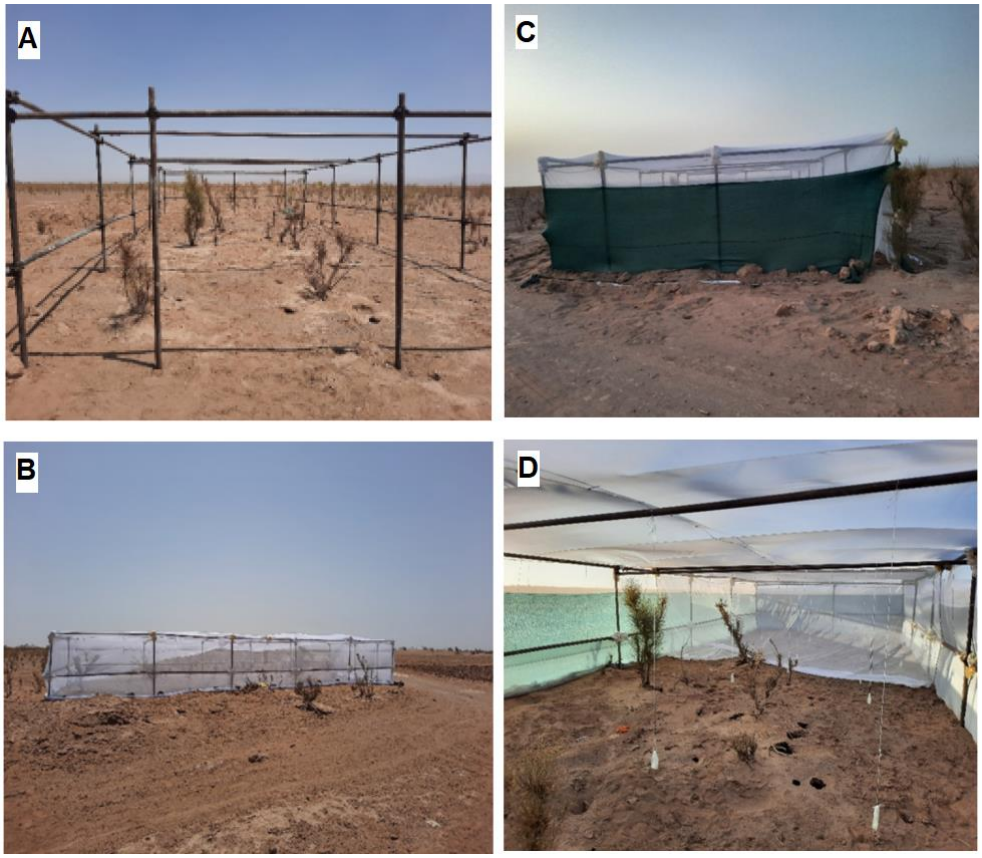

Fig. 2. Vivarium design. A: Framework was installed using scaffolds measuring 15×6×2.5 m, B: The framework was fully covered with a sand fly net with a very fine mesh (mesh number 400) to prevent sand fly escape, C: Green shade net all around the vivarium to protect against wild animals, and D: Interior view of the vivarium in Matin Abad, Badroud, Esfahan Province, central Iran, 2022

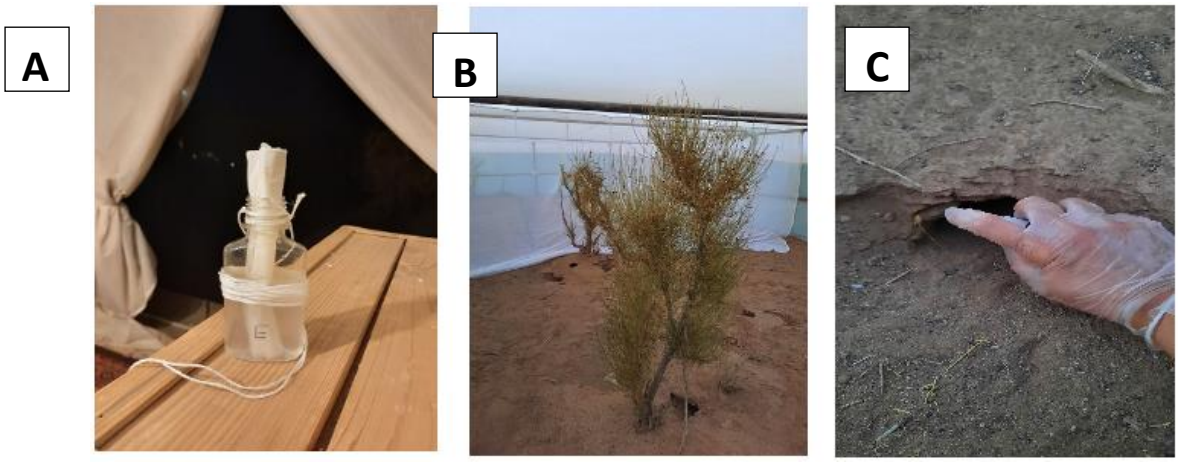

Fig. 3. Three media for delivery of transformed bacteria *Serratia* AS1 and *Enterobacter cloacae* expressing mCherry fluorescent protein. A: Sugar bait, a container filled with 100 ml of PBS-Sugar-Transgenic bacterial suspension, the wick is made of paper towel and a string is present for hanging the container from the scaffold metals. B: *Haloxylon persicum* plant which was sprayed with PBS-Sugar-Transgenic bacterial suspension, C: Rodent burrow soil: 25 ml of PBS-Transgenic bacterial suspension was sprayed in and around the opening of the rodent's burrows in Matin Abad, Badroud, Esfahan Province, central Iran, 2022

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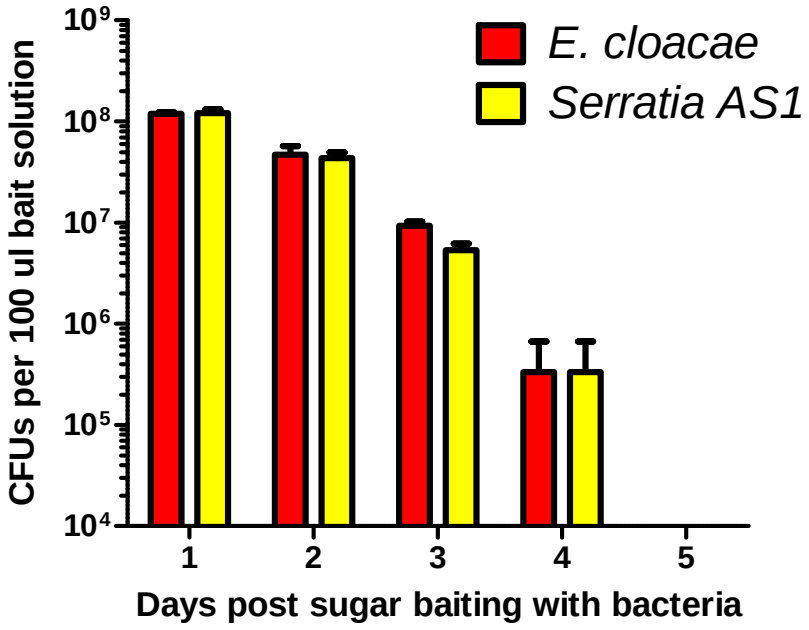

Fig. 4. Stability of two transgenic *Serratia* AS1 and *Enterobacter cloacae* bacteria expressing mCherry fluorescent protein on sugar bait in the contained field (desert) conditions (vivarium) of Matin Abad, Badroud, Esfahan Province, central Iran, 2022. Two-Way ANOVA analysis showed significant differences between days (P< 0.0001) but no significant difference between the bacterial species (P> 0.05). Bars represent Mean ± SEM

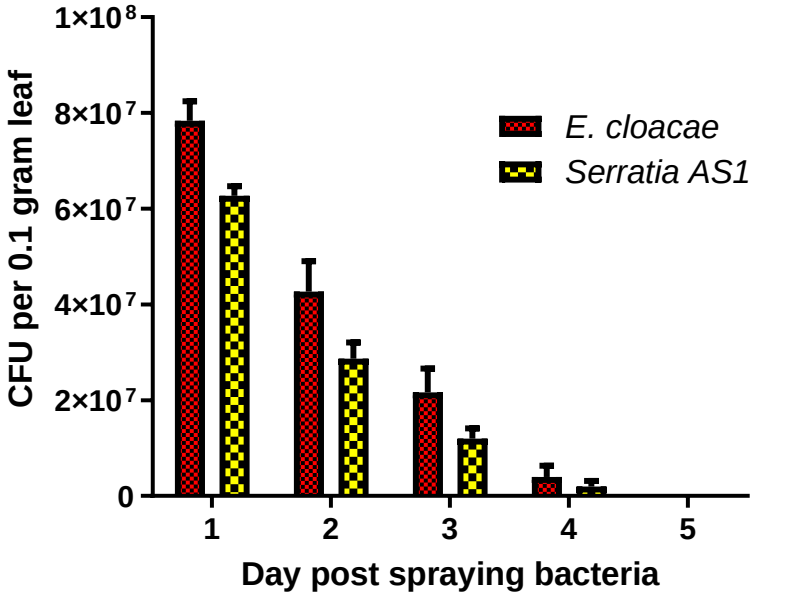

Fig. 5. Stability of two transgenic bacteria *Serratia* AS1 and *Enterobacter cloacae* on the plant leaves of *Haloxylon persicum* in contained field (desert) condition (vivarium), Matin Abad, Badroud, Esfahan Province, central Iran, 2022. Two-Way ANOVA analysis showed that the difference in the number of bacteria between days for both bacteria is significant (P< 0.0001). Also, the differences in the number of bacteria between the two bacteria are significant (P< 0.0001). Bars indicate Mean ± SEM

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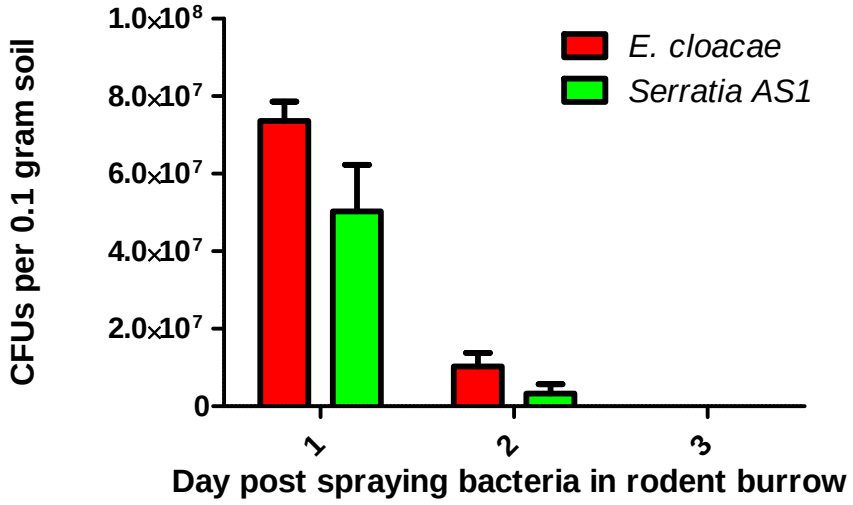

Fig. 6. Stability of two transgenic bacteria, *Serratia* AS1, and *Enterobacter cloacae*, in the soil of rodent burrows in contained-field conditions (vivarium), Matin Abad, Badroud, Esfahan Province, central Iran, 2022. Statistical analyzes (Two-Way ANOVA) showed that the difference in the number of bacteria between days is significant for both bacteria ($P< 0.0001$). The differences in the number of bacteria between the two strains were significant ($P< 0.05$), and the stability of *E. cloacae* bacteria is higher than *Serratia* AS1 on different days ($P= 0.046$). Bars represent Mean ± SEM

Discussion

The stability of transformed bacteria in field conditions is very vital and critical for the paratransgenesis approach. It is worth mentioning that up taking bacteria by the insects may take some time till insects encounter the bacteria after adding the bacteria to the environment. Depending on the biology and physiological characteristics of insects, it may take several hours to a few days, therefore it is very important for the bacteria to be viable and survive in the environment. Harsh environmental conditions including high temperatures and low relative humidity reduce the survival of the bacteria. In addition, among other factors that we did not test here, wind speed, altitude, climate, season, rainfall, sunlight, and the presence of other microorganisms could influence the viability and survival of the bacteria and consequently the success rate of the approach. In the current study, we found that the transformed bacteria remain viable for up to 4 days on the sugar baits and the plant leaves in the harsh desert condition of Matin Abad where the temperature is raised to more than 50 °C in the noon and RH ranges decreased to 10–15%. It is known that if the temperature raises, RH will decrease; thus, the air will become drier and harsher for the survival of the bacteria. It is known that bacteria need moisture or relatively moderate RH to grow (https://www.bbc.co.uk/bitesize/guides/z77v3k7). On the other hand, bacteria have a higher growth rate at 20–30 °C, and the optimal temperature for the growth of bacteria ranges from 23–26 °C (41). In general, the higher the temperature, the more clearly bacteria can grow up to a specified point. Very low and high temperatures both prohibit the enzyme processes of bacteria (42). We suggest that the survival of the bacteria would be more than four days in moderate temperature and RH conditions such as spring or early autumn when sand flies are active in the region. The survival of transgenic bacteria on biotic and abiotic objects plays an important role in the outcome of the paratransgenic approach. The longer the bacteria

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survive on objects, the bigger the chance of the bacteria getting picked up by sand flies.

Delivery of transformed bacteria for the paratransgenesis approach mainly depends on the biology of each vector species. For example, for mosquitoes transmitting malaria, water bodies or larval breeding sites are appropriate sites to introduce the transformed bacteria (15, 24, 26, 34). However, these sites are not suitable for sand fly vectors of ZCL, because they use completely different breeding sites. In this study, we investigated three routes (sugar bait, plant leaves, soil) that could directly or indirectly infect larvae or adult sand flies. The sugar bait could directly infect adult sand flies whereas the plant leaves could directly infect adult sand flies or indirectly infect larval sand flies. Adult sand flies could ingest the bacteria while taking sugar meals from plants. Also, the plants are stored in the tunnel system by rodents and consequently could infect sand fly larval breeding sites. In addition, rodents consume the infected plant leaves, and their infected feces will be a source of the bacteria for infecting sand fly larvae (13). It was shown that some strains of bacteria can pass through the alimentary canal of rodent safely (43). The soil also could infect rodent skin; however, the results of this study showed that soil was not a promising site for delivery of the transformed bacteria for paratransgenesis.

Sugar substances play a very important role in the life of sand flies because these insects always need sugar substances to provide energy for their life activities. In nature, sand flies obtain sugar from plant sap or artificial sugar sources. By absorbing sugars, male and female sand flies get the energy needed to survive and or females to produce eggs, and in its absence, the growth and development of sand flies are not complete (44). In this study, we made sugar materials available to sand flies using 1×PBS containing 10% sucrose with the transgenic bacteria *E. cloacae* and *Serratia* AS1. Our data showed that both transformed bacteria remained viable for four days and a significant relationship was seen between the course of time and decreasing the stability of the bacteria. In another study, the survival rate of *E. cloacae* was evaluated in the water of the larval habitat of *Anopheles stephensi*, and results showed that the bacterial population decreased from 2 x 10^7 on the first day to 2960 bacteria per milliliter on the fourteenth day (27). Also, in a study testing survival of *E. cloacae* in a sugar solution containing 10^9 bacteria per ml in 5% and 2.5% fructose on cotton pads under insectarium conditions, the results showed a 10-fold decrease in the number of bacteria within 11 days (26). Similarly, the same results were seen in laboratory conditions where the survival of bacteria in different concentrations of sucrose had a constant trend until the 4^{th} day and decreased about 10 folds until the 7^{th} day (25). Although it should be noted that different types of bacteria have different abilities to survive in sugar syrup depending on different conditions such as pH, temperature, and sugar concentration (45). It is worth mentioning that although sugar can provide favorable conditions for the growth of bacteria due to the presence of nutrients, the high osmotic pressure of the sugar solution (at high sugar concentrations) can damage bacterial cells. Therefore, it is a fact that an increase in sugar concentration can lead to a decrease in the survival time of bacteria (45).

Using sugar bait for the delivery of transformed bacteria has been tested successfully for some important insect vectors. In a contained-field study, researchers used three separate cages for delivery of *Asia* bacterium expressing green fluorescent protein (*Asaia*^{gfp}) to *An. gambiae* mosquitoes. The cages contained only females, only males, and a combination of males and females. They were fed with cotton soaked in sugar water containing *Asaia*^{gfp} bacteria. On the 12th day after the initiation of the study, 91%, 95%, and 79% of the adults infected with the *Asaia* bacteria were reported respectively in the three cages (46). It has also

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been proven that *Homalodisca vitripennis*, a member of the hemipteran order, could receive the engineered bacterium *Pantoea agglomerans* when fed with PBS containing 2% sugar and the bacterium, marked by the EGFP gene (47).

The survival time of bacteria on plants varies depending on various factors such as the type of bacteria, geographical and environmental conditions, and plant species. Bacterial survival is a complex and multifactorial process that can vary due to the presence of nutrients and moisture on the plant's surface. It is also strongly influenced by environmental factors such as temperature, humidity, and ultraviolet (UV) exposure. Research has shown that plant-associated bacteria, such as *Pseudomonas syringae*, can survive on plant surfaces for days to weeks (48, 49). Also, there are several studies on the survival time of human pathogenic bacteria on plants. For example, in investigating the survival of *E. coli* O157:H7(GFP) on lettuce, it was shown that the bacterium can be traced for at least 20 days after spraying on lettuce (at a temperature of 23 to 26 °C, and this period is directly related to the concentration of the bacteria sprayed on lettuce (50). In similar studies, survival times for *E. coli* Lys9 and *Listeria innocua* on lettuce were obtained up to 28 days (51). Our findings show that the stability of both *E. cloacae* and *Serratia* AS1 bacteria on the plant (*H. persicum*) was maximum of four days. Among the main reasons for the mild stability of the bacteria on plants, we can mention very high temperature, low humidity of the environment at the time of the study, and even the type of plant.

There are several biological, physical, and chemical factors that affect the survival of indigenous and non-indigenous bacteria introduced into the soil that we did not examine here. The three most important factors are the type of clay, the amount of clay, and the intensity of bacteria hunting by soil protozoa. These factors are important when releasing non-engineered and genetically engineered microorganisms (GEMs) into the soil (52). Most of the microorganisms that can survive for a long time in dry soil form special resistant structures such as endospores, cysts, conidia, chlamydospores, or sclerotia. However, some bacteria, although unable to form endospores, can survive in the soil during prolonged droughts (53). The physicochemical properties of the soil can play a very important role in the microbial content of the soil and its survival rate in the soil. Some of the most important properties that can manipulate the population and survival of bacteria in soil are soil texture, pH, moisture content, soil organic matter, host plants, and environment. The effect of soil moisture on microbial activity has been studied and found a positive correlation between the two (54). The pH component can change the abiotic factors of the soil such as nutrients and biological factors such as the biomass of bacteria and fungi. The correlation between pH and bacteria is positive, while the correlation is negative for fungi (55). Although several studies indicate that various human pathogens, including Enterobacteriaceae, can survive for long periods of time on dry surfaces (other than soil) (56, 57), in our study maximum survival time in soil was two days for both bacteria and this difference may be due to the various state of environmental conditions such as UV radiation, extreme heat, and extreme dryness.

Conclusion

Among the three routes investigated for delivery of the transformed bacteria, *Serratia* AS1 and *E. cloacae* expressing mCherry fluorescent protein on desert conditions of the endemic ZCL focus in central Iran, plant leaves of *H. persicum* and sugar bait containing 10% sucrose were the most promising routes to keep the bacteria viable for paratransgenesis approach. However, the bacteria should be sprayed on plants or sugar baits and ought to be replaced with new ones at four days intervals.

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Ethical consideration

This study has been reviewed and approved by the School of Public Health (SPH), Tehran University of Medical Sciences (TUMS) ethics committee and has been registered with the code IR.TUMS.SPH.REC.1399.137

Conflict of interest statement

The authors declare there is no conflict of interests.

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