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Full Length Research Paper

Effects of contaminated irrigation water on the fate of salmonella typhimurium on rosemary and barley cultivated on contaminated fields

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Animal wastes in the form of manure frequently contain enteric pathogenic microorganisms and land spreading can lead to pathogen entry into the food chain. Therefore, the aim of the present study was to determine the persistence of *Salmonella enterica* serovar *typhimurium* in soil, and on barley and rosemary plants. We observed that *Salmonella typhimurium* persisted for an extended period of time (203 to 231 days), and could be detected on infected vegetative parts of the rosemary and barley plants even after desiccation. After approximately two months, the colony morphology displayed a mucoid and rugose phenotype. Smooth colony morphology was acquired following incubation in nutrient broth and upon isolation from the digestive tracts of mice that had been challenged orally with stressed *S. typhimurium*. *S. typhimurium* was neither isolated from vegetative parts formed after plant contamination, nor from barley seeds and rosemary flowers.

Keywords: *Salmonella*, soil, rosemary, barley, contamination.

INTRODUCTION

Animal wastes in the form of manure are largely recycled to agricultural land as the most economical and environmentally sustainable means of treatment and reuse. These materials have a beneficial fertilizer value (nitrogen-phosphate-potassium) and can help maintain soil quality and fertility. However, animal manures frequently contain enteric pathogenic micro- organisms (Pell, 1997) and land spreading can lead to pathogen entry into the food chain. Therefore, controlling the levels of pathogens in animal wastes used on agricultural fields should help to reduce pathogen contamination of soil, surrounding water and produce grown in these areas. Although manure is an obvious source of pathogens, two additional sources of pathogens that contaminate soil, water and crops, and serve as sources of infection for animals and humans are run-off water from manure fields and irrigation water containing manure (Gagliardi and Karns, 2002). Many disease outbreaks have been associated with water or food, including processed fruits and vegetables, which have been directly or indirectly contaminated with animal manure (Cieslak et al., 1993; Van et al., 1999). Cross-contamination of produce from manure or improperly composted manure used on the farm can be a source of pathogen contamination. In this study, our objective was to determine the fate of *Salmonella enterica* serovar *typhimurium* on rosemary and barley plants and in the surrounding soil when contaminated irrigation wa-ter was used. We chose these two plants, since the soil where they are grown is generally fertilized by animal wastes in the form of manure. Our aim was also to determine the length of time that *Salmonella typhimurium* persisted in soil and contaminated plants.

MATERIALS AND METHODS

S. enterica serovar *typhimurium* was kindly provided by A. Bakhrouf, Pharmacy University of Monastir. Cells were thawed from a frozen stock culture, streaked onto Tryptic Soy agar (TSA; Difco Laboratories, Detroit, MI), and incubated for 24 h at 37°C.

Irrigation water was inoculated with 10^9 *Salmonella* serovar *typhimurium* ml^{-1}. Within one month after the barley was seeded, a one-time treatment of two liters of contaminated irrigation water was hand-sprayed onto the soil and barley plants (which were almost five cm in length) of each of the ten plots. We also used ten replicates. Each plot measured 1.8 x 4.6 m. The different plots were protected by a glass cover and exposed to sunlight. The same ex-

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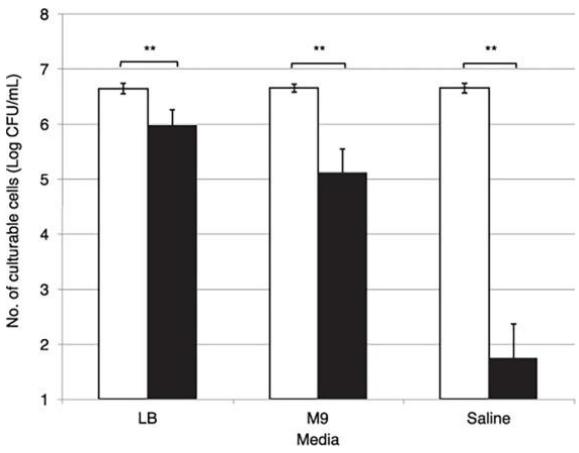

Figure 1. Entrance of logarithmic- phase cells of *Salmonella* Typhimurium into the VBNC state incubated in soil. Shown are the results of total direct count (— —) , CTC viable count (- -), and plate count (—).

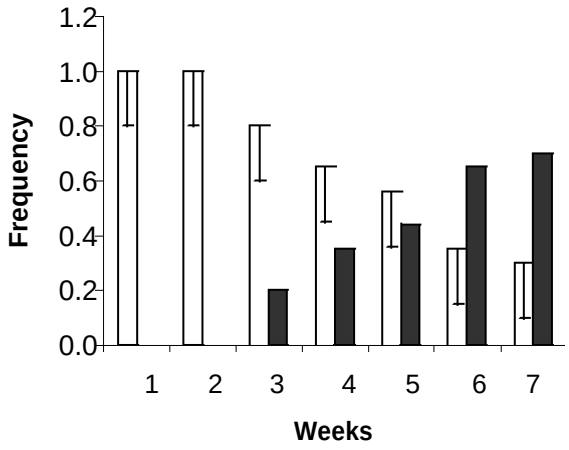

Figure 2: Frequency of Salmonella phenotypes when revived in nutrient broth ( ): rugose colonies.

experimental procedures were carried out with plots planted with rosemary.

At selected time intervals, for each plot, 100 g of soil was aseptically collected with a sterile spoon in a sterile plastic bag from around a randomly selected plant at 2.5 cm below the surface. From each plot, a randomly selected plant was pulled from the soil and collected aseptically in a sterile plastic bag. The samples were transported to the laboratory in a cooler with ice, placed in a walk - in cooler at 4°C within 4 h of collection, and analyzed within 48 h. Each soil sample (10 g) was mixed with 90 ml of 0.1% peptone water in a sterile Whirl-Pak bag and pummeled in a stomacher for 30 s at low speed. Approximately 5 g of each plant (barley or rosemary) was mixed with 45 ml of 0.1% peptone water in a sterile Whirl -Pak bag and rinsed by rubbing and vigorously agitating the plant by hand for 30 s. *S. typhimurium* counts in peptone water of soil samples and in peptone wash water of plant samples were determined. Serial dilutions (1:10) were prepared from each sample, using 0.1% peptone water, and 0.1-ml aliquots of each dilution, in duplicate, were spread onto MacConkey agar containing 1% maltose. Plates were incubated at 37°C for 24 h, and colonies of *S. typhimurium*, which were white with a pink center, were counted. Randomly selected colonies that were white with a pink center were confirmed to be *Salmonella* by a latex agglutination test (Oxoid Inc., Ogdensburg, N.Y.) . Each treatment was repeated five times, and each sample from a treatment was plated in duplicate at each sampling time.

Viable cell counts were determined using the 5-cyano-2,3-ditolyl tetrazolium chloride (CTC; Polysciences Europe, Eppelheim, Germany) method (Rodriguez et al., 1992); that is, bacteria with a functioning electron transport chain reduce the CTC in CTC-formazan, forming a red fluorescent precipitate in the cell membrane. Samples were incubated with 3.0 mM CTC, in the presence of 0.025% yeast extract, at room temperature and then fixed with formalin (2% final concentration). Samples were also stained with DAPI. Cells exhibiting a red precipitate under green excitation were counted. Entry to the viable but nonculturable state (VBNC) was monitored using colony forming unit (CFU) enumeration and total and viable counts, as described above.

RESULTS AND DISCUSSION

Figure 1 shows the responses of *S. typhimurum* to incubation in soil. Three different methods were used to monitor the cells: plate count, DAPI staining and CTC viable count (Cappelier et al., 1997). *S. typhimurium* inoculated into soil displayed declining plate counts and accumulation of large numbers of nonculturable cells. This result has been interpreted in other studies as an indication that nonculturable cells were still viable and thus in the VBNC state (Barer et al., 1993). Figure 1 also shows that *S. typhimurium* could be detected in culturable state even after nine months of starvation in soil. Survival times for up to 300 days in soils spread with cattle slurry have been reported, with survival for up to 259 days having been observed in soils amended with animal faeces (Jones, 1986).

We observed that *S. typhimurium* persisted in different vegetative parts of barley and rosemary plants during the nine moths. During the experiment, every week the different vegetative parts of barley and rosemary were analyzed. We observed that *S. typhimurium* persisted and remained in a cultivable state, but the colony morphology changed after nearly two months to mucoid and rugose phenotypes. Rice et al. (1992) reported that *Vi-brio cholerae* O1 from the Peru epidemic was able to change to a phenotype having wrinkled or rugose colony morphology (Rice et al., 1992). Morris et al. (1996) also reported that *V. cholerae* can change to rugose colony morphology, which is associated with the expression of an amorphous exopolysaccharide (EPS) that promotes cell aggregation. They not only confirmed that rugose strains displayed resistance to killing by chlorine and complement-mediated serum bactericidal activity, but also indicated that these rugose strains cause human disease (Morris et al., 1996). Indeed, compared with cells from smooth variant colonies, rugose cells displayed increased resistance to chlorine, salt and oxidative stress (Wai et al., 1998), and had the ability to form biofilms

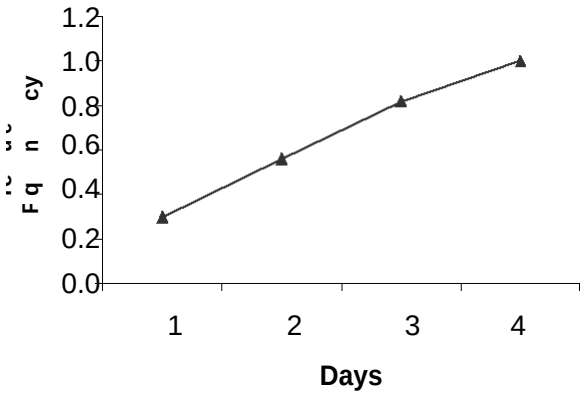

Figure 3. Appearance of smooth *Salmonella* phenotype in mice faeces.

(Mizunoe et al., 1999).

To determine if the mucoid and rugose phenotypes are stably maintained, stressed *S. typhimurium* cells were incubated at 37°C in nutrient broth over two months with shaking, and every 48 h the medium was replenished. Figure 2 showed that following incubation in nutrient broth the smooth colony phenotype was acquired. To determine whether the rugose phenotype is stably maintained, an *in vivo* assay using mice as an animal model was subsequently performed. Twenty groups, comprising of six mice each (six-week-old, male, BALB/c), were obtained from the University of Pharmacy of Monastir and maintained in animal facilities for the duration of the experiment. Ten groups of mice were challenged by oral administration of a 1 ml bacterial suspension, prepared from colonies displaying a rugose colony morphology phenotype. The last group of mice served as a control. The spleens and livers of dead mice were removed for the isolation of culturable *Salmonella*. Faeces were collected daily from all animals, weighed and serially diluted for plating. We observed that the smooth colony phenotype could be acquired in digestive tracts of mice. At one day post-infection, *Salmonella* that resulted in colonies with a smooth phenotype could be isolated from feces and organs (stomach, large and small intestines, liver and lungs) of infected mice. The percentage of colonies displaying a smooth phenotype increased in SS medium with a concomitant disappearance of colonies displaying rugose phenotype after four days (Figure 3). *Salmonella* was not isolated from the faeces and organs of control mice. Nonculturable *V. cholerae* and *Vibrio vulnificus* have been reported to reside in the intestines of rabbits and mice, respecttively (Porter et al., 1980). However, a recent report indicated that NaCl- stressed *Escherichia coli* O157:H7 did not recover in the mouse intestine. The simultaneous loss of culturability and pathogenicity have been observed in *Campylobacter jejuni*, despite a high level of viability in the cellular population tested (Medema et al., 1992).

We observed that *S. typhimurium* remained on infected parts of the different plants studied. Vegetative parts of barley and rosemary formed after contamination did not show any *S. typhimurium*. We did not isolate any *S. typhimurium* from barley seeds and rosemary flowers. Studies have reported that pathogens applied directly to plants survived for shorter periods of time than those applied to soils (Gagliardi, 2002). When manures are applied to land, there is likely to be some movement of the pathogens that they contain through the soil matrix, both vertically and horizontally. The degree of movement will affect the likelihood of pathogens reaching aquifers or surface waters. If these waters are subsequently used for irrigation of produce or for consumption by livestock, there are implications for food safety. Factors known to influence the horizontal movement of pathogens across soils include soil type, soil water content, amount and intensity of rainfall, temperature, nematodal activity, surface charge and size of micro organism, transport through plant roots, and soil pH (Mawdsley et al., 1995, Pike et al., 1986). Factors influencing the vertical movement of pathogens through the soil include the amount and intensity of rainfall, the proximity of the pollutant source, agricultural practice, weather, and the season of application (Mawdsley et al., 1995; Pike et al., 1986). Generally, pathogen survival is favored in aqueous environments, and thus water availability and movement are the single most important factors in determining how far pathogens are likely to move through or across soils. Temperature is also an important consideration, with higher temperatures, e.g., 35°C, reducing pathogen survival (Sorber and Moore, 1987).

In conclusion, we observed in the present study that *S. typhimurium* persisted in soil for an extended period of time (203 to 231 days), and could be detected on infected vegetative parts of the rosemary and barley plants even after desiccation. However, *S.* typhimurium was neither isolated from vegetative parts formed after plant contamination, nor from barley seeds and rosemary flowers. Stressed cells displayed a mucoid and rugose phenotype, but smooth colony morphology was acquired following incubation in nutrient broth and upon isolation from the digestive tracts of mice that had been challenged orally with stressed *S. typhimurium*.

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