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ORIGINAL ARTICLE

Development and validation of a culture-based method suitable for monitoring environmental survival of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in developing countries

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Abstract A non-GMO culture-based method suitable for studying the fate of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in an agricultural environment in developing countries was developed and validated. This method is based on the use of spontaneous rifampicin-resistant mutants of non-toxigenic variants of *E. coli* O157:H7 and *S*. Typhimurium combined with counting of colony-forming units (CFU) on selective media, i.e., Cefixime–Tellurite–Sorbitol MacConkey agar containing 100 μg/ml rifampicin, 50 μg/ml cycloheximide and 50 μg/ml nystatin for *E. coli* O157:H7 and Xylose–Lysine–Tergitol-4 agar containing the same antimicrobials for *S*. Typhimurium. Validation experiments using *gfp*- and *ds-red*-labelled derivatives of the rifampicin-resistant mutants in a non-sterile manure-soil matrix demonstrated that the new culture method was effective in the selection and recovery of the test strains without any detectable interference from background contaminants both in the short and long term. The rifampicin-resistant-based culture method designed provides a feasible low-cost option to study environmental survival of *E. coli* O157:H7 and *Salmonella* spp. in developing countries.

Keywords Culture-based method · Rifampicin-resistant · *Eschericha coli* O157:H7 · *Salmonella* Typhimurium · Environmental survival · Developing countries

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Introduction

Escherichia coli O157:H7 and *Salmonella enterica* serovar Typhimurium are important food-borne pathogens associated with fresh vegetables (Nguyen-the and Carlin 2000; Quiroz-Santiago et al. 2009). Contamination of vegetables in the field with these two pathogens has been attributed to, among other factors, the utilisation of non-composted bovine manure to fertilise agricultural soils (Beuchat 2002), thus prompting the need to establish the fate of pathogens in soils upon manure amendment. The fate of *E. coli* O157:H7 and *S*. Typhimurium as well as other bacterial pathogens in agricultural soils following manure amendment or other matrices is often determined by introducing selected strains of the test organisms into the matrix of interest and following their survival over time. Several

methods ranging from simple culture-based techniques to advanced molecular tools have been used to monitor populations of the introduced test strains (Fratamico et al. 1997; Kudva et al. 1998; Marsh et al. 1998; Ibekwe and Grieve 2003; Ritchie et al. 2003; Artz et al. 2006; You et al. 2006; Novinsack et al. 2007; Semenov et al. 2009). However, for developing countries such as many African countries where vegetable production takes place entirely in the field and fertilisation using manure is often used, a simple low-cost and environmentally-friendly method which can accurately detect and quantify the introduced test organisms in the field is paramount.

Advanced molecular techniques such as quantitative polymerase chain reaction, fluorescent in situ hybridisation and bioluminescence are not relevant for bacterial survival studies in developing countries because such techniques require expensive equipment which is often not available in the ill-equipped laboratories typically found in these countries, let alone the inherent accuracy-related pitfalls associated with practical utility of the techniques (Tebbe and Vahjen 1993; Monterio et al. 1997; van Wintzingerode et al. 1997; Wilson 1997; Ibekwe and Grieve 2003; Ritchie et al. 2003; Artz et al. 2006; Klerks et al. 2006). Current culture-based techniques for detection of *E. coli* O157:H7 and *Salmonella* spp. have practical and environmental limitations. Culture methods that make use of commercial selective media require additional confirmation tests such as genomic DNA finger printing (Kudva et al. 1998), serological tests or biochemical reactions (Lau and Ingham 2001; Natvig et al. 2002; Ingham et al. 2004a) in order to discriminate test strains from background flora, thus making the method still expensive and laborious. Additionally, in case of high populations of background organisms and low numbers of target organisms, visual identification and subsequent enumeration of the target organism becomes difficult (You et al. 2006). The use of strain derivatives containing marker genes such as the green fluorescent protein can eliminate the need for additional confirmation tests in CFU counting (Jiang et al. 2002; Islam et al. 2004a, b; Franz et al. 2005, 2007; Semenov et al. 2007), but restriction on field release of non-approved genetically modified organisms (GMO) jeopardises the applicability of genetically tagged strains in field studies. In respect of regulations regarding the preservation of environmental integrity, there is a need for an alternative non-GMO-culture-based method that can effectively discriminate target organisms from background contaminants. In this study, we present the development and validation of a culture method based on the use of spontaneous rifampicin-resistant mutants and appropriate selective media to detect and quantify *E. coli* O157:H7 and *S.* Typhimurium in agricultural soils.

Materials and methods

Bacterial strains and plasmids

The bacterial strains used were *Salmonella* Typhimurium LT2, *Escherichia coli* O157:H7 ATCC 43888 and *E. coli* S17-1 λ *pir*. *E. coli* S17-1 λ *pir* is a derivative of *E. coli* K12 Tp^r Sm^r *recA thi hsd* RM^+ RP4::2-Tc::Mu::Km Tn7, λ *pir* phage lysogen (De Lorenzo and Timmis 1994) and contains a chromosomally integrated RP4 derivative providing conjugal transfer functions (Simon et al. 1983). The plasmids used were pJBA-28 and pTTN134. pJBA-28 is the delivery plasmid for mini-Tn5-Km-$P_{A1/04/03}$-RBSII-*gfp*mut3*-T_o-T_1 (Andersen et al. 1998) while pTTN134 is the delivery plasmid for mini-Tn5-$P_{A1/04/03}$-RBSII-*dsRed*-T_o-T_1 (Tolker-Nielsen et al. 2000).

Construction of rifampicin-resistant (Rifr) mutants

Salmonella Typhimurium LT2 and *E. coli* O157:H7 ATCC 43888 were made rifampicin-resistant by successive transfer of colonies grown overnight on Luria Bertani (LB) agar containing increasing concentrations of rifampicin from 50 to 150 µg/ml. Mutants resistant (six colonies of each strain) to the antibiotic at a particular concentration were transferred four times to new LB plates with the same antibiotic concentration before transfer to plates with a higher concentration until 150 µg/ml was reached. This was followed by four successive growing overnight of the mutants in LB containing 150 µg/ml rifampicin (LB-Rif150). Rifr mutants of *E. coli* O157:H7 and *S.* Typhimurium were then transferred onto Cefixime–Tellurite–Sorbitol MacConkey agar (CT-SMAC; Merk, Darmstadt, Germany) supplemented with 150 µg/ml rifampicin (CT-SMAC-Rif150), and Xylose–Lysine–Tergitol-4 agar (XLT4; Merk) containing XLT4 supplement and 150 µg/ml rifampicin (XLT4-Rif150), respectively, and the transfers were also repeated four times.

To check for stability of the Rifr marker in the mutants, six mutants of each strain were separately grown overnight at 37°C in 50 ml of LB-Rif150. The cultures were washed 3 times in 0.9% NaCl and diluted to an optical density (OD_{650}) of 0.05. Then, 10 µl of the diluted suspension were transferred to 50 ml of fresh LB broth without rifampicin and incubated with agitation (150 rpm) at 37°C until an OD_{650} of 0.7. The cultures were then adjusted to an OD_{650} of 0.05 using 0.9% NaCl, and 10 µl of this suspension were transferred to 50 ml of fresh LB and incubated with agitation (150 rpm) at 37°C until OD_{650} of 0.7. This was followed by 8 successive transfers in LB without rifampicin using the same procedure after which the cultures (corresponding with approximately 180 generations in total) were serially diluted in 0.9% NaCl followed by

plating in duplicate 100 μl of appropriate dilutions on LB, LB-Rif100 and LB-Rif150. CFU were enumerated after 24 h of incubation at 37°C.

To check the recovery of Rifr-mutants on solid media, six mutants of each strain taken from CT-SMAC-Rif150 or XLT4-Rif150 plates were separately grown in LB-Rif150 to OD_{650} of 0.7. The cells were washed in 0.9% NaCl, diluted, and inoculated in fresh LB-Rif150 and in LB at initial OD_{650} of 0.05. The cells were grown at 37°C with agitation (150 rpm) until OD_{650} of 0.7 followed by serial dilution in 0.9% NaCl. The Rifr *E. coli* O157:H7 cultures were plated in duplicate on LB, LB-Rif100, LB-Rif100 incorporated with 50 μg/ml cycloheximide and 50 μg/ml nystatin (LB-Rif100-Cy50-Ny50), LB-Rif150, CT-SMAC, CT-SMAC-Rif100, CT-SMAC-Rif100 incorporated with 50 μg/ml cycloheximide and 50 μg/ml nystatin (CT-SMAC-Rif100-Cy50-Ny50) and CT-SMAC-Rif150, respectively. The Rifr *S*. Typhimurium cultures were plated on XLT4, XLT4-Rif100, XLT4-Rif100 incorporated with 50 μg/ml cycloheximide and 50 μg/ml nystatin (XLT4-Rif100-Cy50-Ny50) and XLT4-Rif150 in addition to LB, LB-Rif100, LB-Rif100-Cy50-Ny50 and LB-Rif150. The CFU were enumerated following 24 h of incubation at 37°C.

To check whether the Rifr mutation affected their growth, the growth rates of Rifr-mutants in LB and in LB-Rif100 or LB-Rif150 were compared with the growth rates of the parent strains in LB medium at 37°C. Cultures of parent and six mutants of each strain were separately grown in 50 ml of LB broth contained in 250-ml Erlenmeyer flasks. The initial population density of each culture was approximately 3 log CFU/ml. The cultures were incubated at 37°C with agitation (150 rpm). At specific time intervals (0, 2, 4, 6, 8, 10, 12 and 24 h), a 1-ml sample was removed from each flask, diluted in 0.9% NaCl solution, followed by plating in duplicate appropriate dilutions on LB. The plates were incubated aerobically for 24 h at 37°C and the organisms enumerated.

Construction of genetically labelled-derivatives of Rifr-mutants

Fluorescently labelled derivatives of Rifr *E. coli* O157:H7 and Rifr *S*. Typhimurium were constructed by introducing the gene encoding for the red fluorescent protein (*ds-red)* and green fluorescent protein gene (*gfp*), respectively. Rifr *S*. Typhimurium was labelled with the *gfp* gene by bi-parental mating with pJBA-28 followed by selection of transconjugants on minimal medium supplemented with glucose (20 mg/l), kanamycin (50 μg/ml) and rifampicin (150 μg/ml) (MMG-KR). Transconjugants were checked for green fluorescence expression using an Olympus BX51 epifluorescence microscope (Olympus Optical, Japan). Six green fluorescent mutants were transferred four times on minimal medium and finally to XLT4 containing 50 μg/ml kanamycin and 150 μg/ml rifampicin (XLT4-KR). Typical *Salmonella* colonies growing on XLT4-KR plates (black) were re-checked for green fluorescence expression as mentioned above. Rifr *E. coli* O157: H7 was labelled with *ds-red* gene by bi-parental mating with *ds-red* labelled-*E. coli* S17-1λ*pir* harbouring pTTN134 followed by selection of the transconjugants (red colonies) on MMG-KR. Six transconjugants were transferred four times on MMG-KR and finally to CT-SMAC supplemented with 50 μg/ml kanamycin and 150 μg/ml rifampicin (CT-SMAC-KR). Colonies growing on CT-SMAC-KR were examined for red fluorescence expression by epifluorescence microscopy.

To examine whether the *gfp* or *ds-red* gene was stably inherited by recipient organisms, liquid cultures of six transconjugant strains of *E. coli* O157:H7-Rifr-*ds-red* and *S*. Typhimurium -Rifr-*gfp* were grown overnight at 37°C in 50 ml of LB medium with and without kanamycin (50 μg/ml) selection. Then, 10 μl of the respective cultures were transferred to fresh LB broth and incubated until ~20 generations. Following transfer, 100 μl of each culture was diluted in 0.9% NaCl, plated on LB agar with and without kanamycin (50 μg/ml) and incubated for 24 h at 37°C. The colonies that developed on the plates were checked for fluorescence expression as described before. Successive subculturing and subsequent confirmation of colony fluorescence were performed for up to 180 generations. Finally, to check whether the introduction of *ds-red* or *gfp* gene induced additional metabolic burden on the mutants, the growth rates of the transconjugants were compared with the growth rates of parent strains as described for the Rifr-mutants.

Design of selection media to recover Rifr-mutants from non-sterile matrix

Performances of different media formulations to select and recover Rifr-mutants from mixed microbial populations were evaluated using genetically modified derivatives of Rifr-mutants introduced into non-sterile manure-soil matrix. Two Kenyan soils, classified as Endohypostagnic Lixisol and Geric-Lixic ferralsol (FAO-ISRIC-ISSS 1998), obtained from the villages of Teso and Nyabeda, respectively, were used. Physico-chemical properties of the two soils have been presented elsewhere (Pypers et al. 2006). Fresh bovine manure was obtained from Galloway beef cattle grazing on a meadow. Total aerobic plate counts on LB (mean ± SE) were 6.70±0.56, 6.33±0.93, and 8.74±1.20 log CFU/g for Teso, Nyabeda soil, and manure, respectively. The following agar-based media formulations were evaluated for detection of *E. coli* O157:H7-Rifr-*ds-red*: CT-SMAC, CT-SMAC-Rif100 and CT-SMAC-Rif100-Cy50-Ny50. For detection of *S.* Typhimurium-Rifr-*gfp*, the

following media formulations were evaluated: XLT4, XLT4-Rif100 and XLT4-Rif100-Cy50-Ny50.

To evaluate the capacity of each media formulation to select against background flora present in the manured soil matrix, three replicate samples of 2 g of the manured soil matrix were serially diluted in 0.9% NaCl. Next, 100 µl of the 10^{-1} dilution was plated in duplicate on each of the listed media formulation followed by incubation of the plates for 24 h at 37°C. The colonies that developed on the plates were counted and checked for fluorescence using an Olympus BX51 epifluorescence microscope (Olympus). The manure–soil matrix was then spiked separately with *E. coli* O157:H7-Rifr-*ds-red* and *S.* Typhimurium-Rifr-*gfp* at approximately 7 log CFU/g and immediately sampled for detection of test organisms as described above. XLT4-Rif100-Cy50-Ny50 and CT-SMAC-Rif100-Cy50-Ny50 exhibited 100% selectivity for *S.* Typhimurium-Rifr-*gfp* and *E. coli* O157:H7-Rifr-*ds-red*, respectively. To evaluate the suitability of XLT4-Rif100-Cy50-Ny50 and CT-SMAC-Rif100-Cy50-Ny50 to suppress background flora and to select Rifr-mutants during long-term survival, 1 kg of inoculated as well as non-inoculated (control) manure–soil matrices were dispensed in 2-L plastic pots and incubated for 5 weeks in a screen house designed to simulate tropical field conditions (temperature: 18–38°C; relative humidity: 63–82%). The experiment was replicated three times. The pots were weighed after every other day and water added when necessary to compensate for moisture loss. Three replicate samples of 2 g each were taken weekly from the top (<1 cm deep) and middle (5–6 cm deep) of each matrix, serially diluted in 0.9% NaCl followed by plating in duplicate appropriate dilutions on XLT4-Rif100-Cy50-Ny50 or CT-SMAC-Rif100-Cy50-Ny50. The plates were incubated for 24 h at 37°C followed by CFU counting of the target organism. To confirm whether the colonies on the plates were indeed the introduced test organisms, colonies on a few selected plates were taken for microscopic examination of fluorescence as described before. At each sampling moment, 5-g samples of non-inoculated matrices were taken for the determination of moisture content as previously described by Franz et al. (2005). Moisture profile of the matrices during the 5-week incubation period is shown in Fig.1.

Data analysis

Maximum specific growth rates (which, by definition, correspond to the slope of a plot of the natural logarithm of the bacterial population versus time and has units h^{-1}) of mutants were compared with that of their parent strains by fitting growth data to the MicroFit© software version 1.0 (Institute of Food Research, Norwich, UK). Recovery rate of each media formulation for the test organism was determined by comparing CFU numbers recovered on the test plate with CFU numbers on LB using one-way Analysis of Variance at 5% level of significance and separating the means by LSD method. The ANOVA procedure and mean comparison was done using the S-plus statistical software version 6.1 (www.insightful.com).

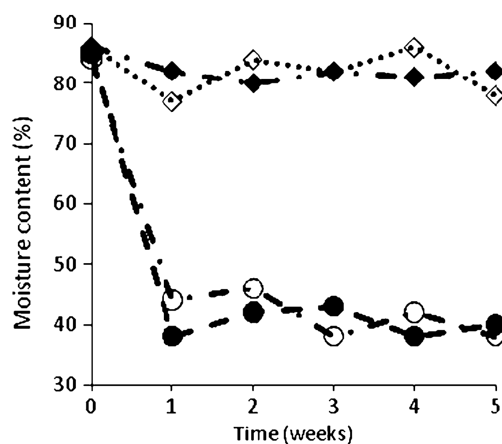

Fig. 1 Moisture content (on dry weight basis) of manure–soil matrix during 5 weeks of incubation in the screen house. (o) Nyabeda soil, matrix sampled from the top; (•) Teso soil, matrix sampled from the top; (◇) Nyabeda soil, matrix sampled from the middle; (♦) Teso soil, matrix sampled from the middle

Results

Construction of Rifr-mutants and genetically-labelled derivatives

Rifr mutants of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium were successfully constructed. Six mutants were retained and examined for stability of the Rifr marker. After 8 successive transfers on non-selective medium (corresponding to approximately 180 generations), the population size (mean log CFU/ml ± SE) of the Rifr *E. coli* O157:H7 mutants in the final culture was identical when counted on LB (9.42±0.77) and LB-Rif100 (9.65±0.43) but significantly lower when counted on LB-Rif150 (7.44±0.53 log). Similar results were obtained for the Rifr *S.* Typhimurium mutants tested (9.75±0.06 log CFU/ml on LB; 9.68±0.16 log CFU/ml on LB-Rfi100; 7.20±0.09 log CFU/ml on LB-Rif150). These results indicate that the Rifr marker was stably inherited by the constructed strains but that the concentration of Rif used in the selective plates affected the recovery. The effect of rifampicin concentration on the recovery of Rifr-mutants on solid media was tested using overnight cultures of the mutants. Results are shown in Table 1. There were no significant differences between counts on LB and on LB-Rif100, CT-SMAC and CT-SMAC-Rif100 for the recovery of the Rifr *E. coli* O157:H7 mutants. On the

Table 1 Recovery of *Escherichia coli* O157:H7-Rifr and *Salmonella* Typhimurium-Rifr on different media formulations

Selective medium	Cell population recovered (log CFU/g)	
	E. coli O157:H7-Rifr	*S.* Typhimurium-Rifr
LB	9.58±0.04 a	9.60±0.12 a
LB-Rif100	9.48±0.14 a	9.70±0.10 a
LB-Rif150	8.39±0.08 b	8.50±0.12 b
LB-Rif100-Cy50-Ny50	9.53±0.22 a	9.68±0.05 a
XLT4	NDD	9.57±0.06 a
XLT4-Rif100	NDD	9.62±0.06 a
XLT4-Rif100-Cy50-Ny50	NDD	9.64±0.07 a
XLT4-Rif150	NDD	8.38±0.05 b
CT-SMAC	9.61±0.10 a	NDD
CT-SMAC-Rif100	9.55±0.05 a	NDD
CT-SMAC-Rif100-Cy50-Ny50	9.60±0.08 a	NDD
CT-SMAC-Rif150	8.52 ±0.15 b	NDD

Values show means ± SE (n=12). Means in the same column followed by different letters are significantly different ($P \leq 0.05$). Detection limit: 2 log CFU/ml

LB Luria-Bertani; *CT-SMAC* Cefixime-Tellurite-Sorbitol MacConkey agar; *XLT4* Xylose-Lysine-Tergitol-4 agar; *Rif100* 100 μg/ml rifampicin; *Rif150* 150 μg/ml rifampicin; *Cy50* 50 μg/ml cycloheximide; *Ny50* 50 μg/ml nystatin; *NDD* not determined

other hand, the population of Rifr *E. coli* O157:H7 on LB-Rif150 and on CT-SMAC-Rif150 were significantly less by approximately 1.2 logs ($P \leq 0.05$). Similar results were obtained for the Rifr *S.* Typhimurium mutants. CFU were not significantly different on LB, LB-Rif100, XLT4 and XLT4-Rif100 ($P > 0.05$) but significantly lower by approximately 1 log on LB-Rif150 and XLT4-Rif150 ($P \leq 0.05$). These results confirm that the concentration of Rif in the medium affected the recovery of the mutant strains.

The growth rates of the six Rifr-mutants of both *S.* Typhimurium and *E. coli* O157:H7 were compared with those of the respective parent strains in order to examine whether the introduced mutation affected growth. Results are shown in Table 2. The growth rates of both Rifr-mutants in LB and in LB-Rif100 were not significantly different from those of their parent strains in LB ($P > 0.05$). However, Rifr *E. coli* O157:H7 and Rifr *S.* Typhimurium grew significantly slower in LB-Rif150 than their parent strains in LB ($P \leq 0.05$).

Single selected Rifr mutants of both *E. coli* O157:H7 and *S.* Typhimurium were retained and designated as *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr, respectively. Gfp and Ds-red marked variants of the *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr strains were successfully constructed. The genetic constructs were brightly fluorescent and the fluorescence was stable for up to 180 generations in non-selective medium as tested for six variants of each strain. The growth rates of *E. coli* O157:H7-Rifr-*ds-red* and *S.* Typhimurium-Rifr-*gfp* mutants in LB and LB-Rif100 were not significantly different from those of their respective parent strains in LB medium ($P > 0.05$) (Table 2). However, the genetic mutants grew significantly slower in LB-Rif150. In addition, recovery on LB and LB-Rif100 was identical. These results show that the Rifr-mutants and their genetic derivatives had the same growth characteristics, which justified the suitability of the genetic derivatives for the design and validation of selective medium for CFU counting of the Rifr-mutants.

Table 2 The effect of antibiotic resistance and genetic marking on maximum specific growth rate of *E. coli* O157:H7 and *S.* Typhimurium

Organism	Growth rate (h^{-1})				
	Wild-type in:	Mutants in:			
	LB		LB	LB-Rif100	LB-Rif150
E. coli O157:H7	1.19±0.04 a	Rifampicin-resistant mutant	1.21±0.01 a	1.22±0.02 a	0.48±0.03 b
		Genetic construct	1.20±0.02 a	1.18±0.03 a	0.56±0.04 b
S. Typhimurium	1.16±0.02 a	Rifampicin-resistant mutant	1.14±0.03 a	1.16±0.05 a	0.69±0.02 b
		Genetic construct	1.18±0.02 a	1.17±0.03 a	0.62±0.07 b

Values show mean ± SE (n=12). Means in the same row followed by different letters are significantly different ($P \leq 0.05$)

LB Luria-Bertani broth; *LB-Rif100* LB containing 100 μg/ml rifampicin; *LB-Rif150* LB containing 150 μg/ml rifampicin

Finally, recovery of one mutant of each constructed strain was also tested on CT-SMAC-Rif100-Cy50-Ny50 for *E. coli* O157:H7 and on XLT4-Rif100-Cy50-Ny50 for *S.* Typhimurium, since these media formulations were selected for selective counting of the strains after addition in non-sterile environmental samples. Recovery on all media compared to LB and LB-Rif100 was in all cases 100% (Table 1).

Selection media for recovery of Rifr-mutants from non-sterile matrix

No fluorescent background organisms were detected in the manure–soil matrix used in this study. Non-inoculated manure–soil matrix was first used to determine the capacity of various media formulations to select against background organisms and the results are shown in Table 3. Average CFU numbers of background organisms on CT-SMAC and on XLT4 were above 3 log CFU/g and consisted of both fungi and bacteria, phenotypically. This level of background counts would make counting of test organisms difficult when present at levels below 3 log CFU/g. On XLT4-Rif100 and CT-SMAC-Rif100 plates, background bacterial CFU were eliminated but fungal colonies were still present above the detection limit of the plating technique (2 log CFU/g), and in some cases fungal mats covered the plates which would still make counting of the test organisms difficult. To eliminate background fungal colonies, a combination of Cy50 and Ny50 needed to be added to the CT-SMAC-Rif100 and XLT4-Rif100, and the resultant media formulations (CT-SMAC-Rif100-Cy50-Ny50 and XLT4-Rif100-Cy50-Ny50) were selected for validation. Recovery of the constructed *E coli* O157:H7-Rifr and *E. coli* O157:H7-Rifr-*ds-red* on CT-SMAC-Rif100-Cy50-Ny50, and *S.* Typhimurium-Rifr and *S.* Typhimurium-Rifr-*gfp* on XLT4-Rif100-Cy50-Ny50 compared to LB and LB-Rif100 was in all cases 100% (Table 1).

Table 3 Capacity of various media formulations to select against background organisms from manure-soil matrix

Selective medium	Recovered cell population (log CFU/g)
XLT4	3.44±0.12[a]
XLT4-Rif100	2.67±0.05[b]
XLT4-Rif100-Cy50-Ny50	BD
CT-SMAC	3.73±0.09[a]
CT-SMAC-Rif100	2.28±0.10[b]
CT-SMAC-Rif100-Cy50-Ny50	BD

Values show mean ± SE (n=6)

CT-SMAC Cefixime-Tellurite-Sorbitol MacConkey agar; *XLT4* Xylose-Lysine-Tergitol-4 agar; *Rif100* 100 μg/ml rifampicin; *Cy50* 50 μg/ml cycloheximide; *Ny50* 50 μg/ml nystatin; *BD* below detection limit (2 log CFU/g)

[a] Fungal and bacterial colonies

[b] Typically fungal colonies

The performance of CT-SMAC-Rif100-Cy50-Ny50 and XLT4-Rif100-Cy50-Ny50 to select and recover the populations of *E. coli* O157:H7-Rifr-*ds-red* and *S.* Typhimurium-Rifr-*gfp*, respectively, from non-sterile manure–soil matrix was then tested and the results are shown in Table 4. Mean CFU counts of *E. coli* O157:H7-Rifr-*ds-red* on CT-SMAC-Rif100-Cy50-Ny50 and of *S.* Typhimurium-Rifr-*gfp* on XLT4-Rif100-Cy50-Ny50 were not significantly different from the respective initial inoculum density introduced into the matrix ($P \leq 0.05$), thus signifying 100% recovery. All the recovered colonies were brightly fluorescent, confirming that all were derived from the constructed *E. coli* O157:H7-Rifr-*ds-red* and *S.* Typhimurium-Rifr-*gfp* strains, and hence validating the suitability of CT-SMAC-Rif100-Cy50-Ny50 and XLT4-Rif100-Cy50-Ny50 to recover Rifr mutants from non-sterile environmental matrices.

Finally, the suitability of CT-SMAC-Rif100-Cy50-Ny50 and XLT4-Rif100-Cy50-Ny50 to monitor the population of Rifr-mutants in the long term was demonstrated during a 5-week incubation of manured soil matrices inoculated with *E. coli* O157:H7-Rifr-*ds-red* or *S.* Typhimurium-Rifr-*gfp*. Survival patterns of *E. coli* O157:H7-Rifr-*ds-red* and *S.* Typhimurium-Rifr-*gfp* depended on sample location (top versus middle). In the middle of the matrices, CFU numbers of *E. coli* O157:H7-Rifr-*ds*-red and *S.* Typhimurium-Rifr-*gfp* stabilised for 1 week after which cell counts of the organisms declined with time (Fig. 2). On the top of the matrices, the population sizes of both organisms declined with time and there was no evidence of an initial delay as observed in the case of samples taken from the middle of the matrices. *E. coli* O157:H7-Rifr-*ds-red* and *S.* Typhimurium-Rifr-*gfp* survived better in the middle than on the top of the matrices regardless of the soil type. Irrespective of sample location (top or middle) and sampling time, CT-SMAC-Rif100-Cy50-Ny50 and XLT4-Rif100-Cy50-Ny50 selected *E. coli* O157:H7-Rifr-*ds-red* and *S.* Typhimurium-Rifr-*gfp*, respectively, without interference from background organisms, and all the colonies examined were brightly fluorescent, hence validating the utility of the new culture method for long-term survival studies.

Discussion

In this study, we successfully constructed Rifr-derivatives of non-toxigenic strains of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium and designed appropriate selec-

Table 4 Suitability of XLT4-Rif100-Cy50-Ny50 and CT-SMAC-Rif100-Cy50-Ny50 formulations for selective recovery of test organisms from manure-soil matrix

Selective medium	Recovered cell population (log CFU/g)		
	Inoculated with		Control
	E. coli O157:H7 Rif-*ds-red*[a]	*S.* Typhimurium Rif-*gfp*[b]	
XLT4-Rif100-Cy50-Ny50	NDD	7.62±0.18	BD
CT-SMAC-Rif100-Cy50-Ny50	7.53±0.07	NDD	BD

Values show mean ± SE (n=6)

Control non-spiked manure-soil matrix; *CT-SMAC* Cefixime-Tellurite-Sorbitol MacConkey agar; *XLT4* Xylose-Lysine-Tergitol-4 agar; *Rif100* 100 μg/ml rifampicin; *Cy50* 50 μg/ml cycloheximide; *Ny50* 50 μg/ml nystatin; *BD* below detection limit (2 log CFU/g); *NDD* not determined

[a] Introduced into the matrix at 7.66±0.11 log CFU/g

[b] Introduced into the matrix at 7.70±0.05 log CFU/g

tion media for their enumeration in environmental matrices. Non-pathogenic strains were used because of public health regulations in many countries which have few laboratories below hazard level 3 status for conducting research using toxigenic strains of pathogenic organisms (Ritchie et al. 2003; Artz et al. 2006). The use of non-toxigenic strains to simulate the behaviour of pathogenic organisms in survival studies is appropriate and has been shown previously to be adequate for the case of *E. coli* O157:H7, where studies designed to compare the survival of toxigenic and non-toxigenic strains of the organism in animal wastes showed identical survival patterns (Kudva et al. 1998).

The fact that there were no significant differences between the growth rates of the Rifr-mutants in LB and in LB-Rif100 broth, when compared with the growth rates of their parent strains in LB (Table 2), implies that 100 μg/ml rifampicin did not induce a tremendous metabolic burden on the organisms. However, decreased growth rates of Rifr-mutants in LB-Rif150 compared to those recorded in LB and LB-Rif100 shows that 150 μg/ml rifampicin affected metabolism in the mutant strains, despite the fact that *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr were initially developed to resist up to 150 μg/ml rifampicin on solid media. The unsuitability of the 150 μg/ml rifampicin for use in the medium was further shown in the recovery tests where cell counts of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr were grossly underestimated on plates containing 150 μg/ml rifampicin (Table 1). These findings provide some lessons to the effect that, for practical purposes, it might be of interest to develop the resistant mutants at antibiotic concentrations well above the intended concentration for use. Based on these findings, we can conservatively assert that the mutants effectively maintained and sustained resistance to rifampicin both on solid and in liquid media up to a concentration of 100 μg/ml.

The *gfp*- and *ds-red*-labelled mutants were appropriate for use in the design of selection media for two reasons: first, the growth rates of genetically labelled mutants were not significantly different from those of their parent strains, as was the case for the Rifr-derivatives in LB and in LB-Rif100; and secondly, genetically labelled mutants had stable insertion of the *ds-red* and *gfp* gene, respectively,

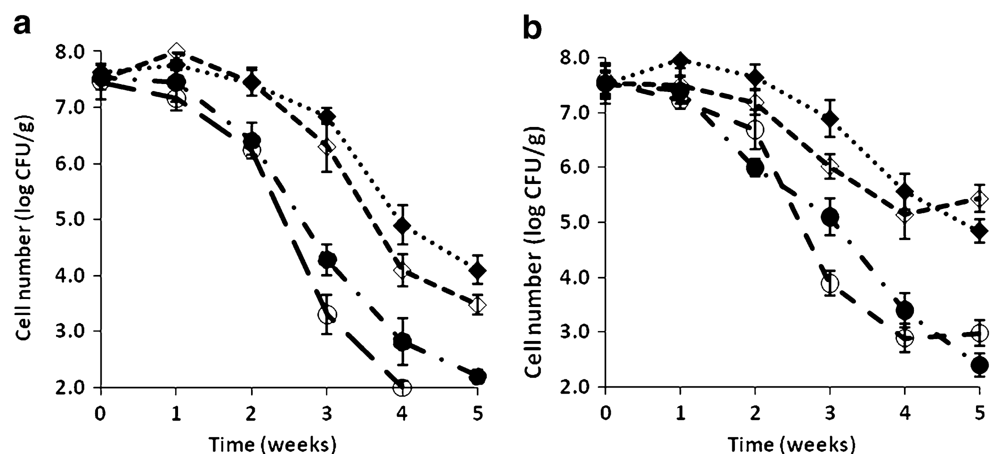

Fig. 2 Survival of *E. coli* O157:H7-Rifr-*ds-red* (**a**) and *S.* Typhimurium-Rifr-*gfp* (**b**) in manure–soil matrix during 5 weeks of incubation in the screen house. (o) Nyabeda soil, matrix sampled from the top; (•) Teso soil, matrix sampled from the top; (⋄) Nyabeda soil, matrix sampled from the middle; (♦) Teso soil, matrix sampled from the middle. Data points show mean ± SE (n=18)

into their chromosome which enabled colony verification by epifluorescence microscopy without the need for application of the kanamycin antibiotic marker. The characteristic straw-like and black-coloured phenotypic appearance of *E. coli* O157:H7 and *Salmonella* on CT-SMAC and XLT4 was the basis for the choice of CT-SMAC and XLT4, respectively, for use in the design of selective medium. It was envisaged that a combination of these phenotypic characteristics and 100 μg/ml rifampicin would provide effective criteria for selective detection and enumeration of Rifr-mutants in mixed microbial populations. Inclusion of antifungal agents (cycloheximide and nystatin) at concentrations of 50 μ/ml each in the selection media proved needed to satisfactorily control interference from background fungi, while 100 μg/ml rifampicin was effective to eliminate background bacterial contaminants. During construction of Rifr-mutants, it was observed that respective populations of *E. coli* O157:H7-Rifr and S. Typhimurium-Rifr on CT-SMAC-Rif100-Cy50-Ny50 and XLT4-Rif100-Cy50-Ny50 were the same as observed on non-selective medium (LB). Furthermore, CT-SMAC-Rif100-Cy50-Ny50 and XLT4-Rif100-Cy50-Ny50 efficiently selected *E. coli* O157:H7-Rifr-*ds-red* and *S.* Typhimurium-Rifr-*gfp* from manure–soil matrix, respectively. These results demonstrate that CT-SMAC-Rif100-Cy50-Ny50 and XLT4-Rif100-Cy50-Ny50 formulations would be most suitable for the selection and recovery of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr mutants during survival studies. This was explicitly shown in a survival experiment where fluorescent marked derivatives of the strains were used to confirm the identity of the introduced strains in the matrix after plating. It was interesting to note that both *E. coli* O157:H7-Rifr-*ds-red* and *S.* Typhimurium-Rifr-*gfp* declined faster on the top than in the middle of the matrices during the 5-week incubation period (Fig. 2). This was probably due to lower moisture level on the former than in the latter location (Fig.1), and is consistent with the findings of Kudva et al. (1998) on the survival of *E. coli* O157:H7 in cattle manure and those of Himathongkham et al. (1999) on the survival of *S.* Enteritidis and *S.* Typhimurium in chicken manure.

In other studies where commercial selective media such as XLD and CT-SMAC were solely employed to recover the populations of non-genetically marked organisms as test strains (Hutchison et al. 2004, 2005; You et al. 2006), the authors did not demonstrate the suitability of the culture media to recover the populations of the test organism introduced into the matrix, hence they could not show how the populations of test strains were discriminated from background organisms. For instance, it is well known that some other bacterial species are sorbitol-negative and therefore exhibit the same phenotypic characteristic as *E. coli* O157:H7 on CT-SMAC agar (Fratamico et al. 1993). Creation of Rifr-derivatives as done in this study therefore provides useful markers for application in survival studies using a culture-based method when combined with antifungal agents and further avoids the need for additional confirmation experiments. We do acknowledge that use of culture-based methods to study environmental survival of bacterial pathogens has been excoriated by proponents of the viable but non-culturable (VBNC) physiological state of bacteria (Ritchie et al. 2003), albeit controversies surround the VBNC phenomenon (Barer et al. 1993; Bogosian et al. 1998; Kell et al. 1998; Oliver 2005; Dinu et al. 2009, Dhiaf et al. 2010). But nevertheless, we believe that the culture method developed in this work is a feasible and effective option to study environmental survival of *E. coli* O157:H7 and *Salmonella* spp. in developing countries.

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