Tetracycline efflux pump in different *Salmonella enterica* isolated from diarrhea patients in two rural health centers in Western Kenya

Onyango David Miruka[1*], Kakai Rose[2], Waindi Eliud Nyandago [1]

[1] *Department of Zoology, Maseno University, Maseno, Kenya*

[2]*School of Public Health and Community Development, Maseno University, Maseno, Kenya*

ABSTRACT

Background: Tetracyclines (TCs) are a type of broad-spectrum bacteriostatic antibiotics used for treatment of a wide variety of infections. These antibiotics eventually reach terrestrial and aquatic environments via application of manure or slurry to areas used agriculturally, or by other methods. Bacteria resistance to tetracycline is due to efflux pump among others. This study discerned the existence of tetracycline efflux pump in *Salmonella enterica* belonging to different serovars isolated from diarrhea patients in two rural health centers in Western Kenya.

Patients and method: Forty-five unrelated tetracycline-resistant *Salmonella* isolates from diarrheal patients were cultured and phenotypically identified using conventional methods. Phenotypic tetracycline resistance profile was determined by using Agar Disc Dilution. Tetracycline growth curve for resistant isolates were determined by incubating 10^6 cfu in 10 ml nutrient broth having 30µg/ml tetracycline and incubated at 37°C in a rotary incubator. Generation rate was spectrophotometrically assayed at 600nm. Tetracycline resistant isolates DNA were extracted using QIAMP Qiagen protocol and amplified using specific tetracycline primers.

Results: Tetracycline resistance genes were isolated in 62.2% (n=28/45) *Salmonella* isolates. Out of these, 64.3% (n =18/28) were *S. typhi*, 17.8% (n =5/28) *S. typhimurium*, and 14.3% (n=4/28) *S. enteritidis*. *S. typhi* expressed resistance to *tet*A 3.6% (n=1/28), *tet*B 10.7% (n=3/28), *tet*D 10.7% (n=3/28), *tet*G 10.7% (n=3/28), *tet*E, H, J, 10.7% (n=3/28) and *tet* AC 17.8% (n=5/28) in isolation or in combination. All the 5 *S. typhimurium* expressed *tet*A resistance. Totally, *S. enteritidis* displayed *tet*A, *tet*B and *tet*C resistance in 7.1%, 3.6%, and 3.6%, respectively.

Conclusion: These data show that the identified gene encoded *tet* resistance gene as MFS–type multi-substrate efflux transporter, which contributes to intrinsic resistance on range of structurally unrelated compounds in *Salmonella* species.

Keywords: *Colony forming unit (cfu); Tetracycline; Resistance; Efflux pump*.
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INTRODUCTION

Although tetracyclines are the second most commonly used antibiotics worldwide, their efficacy has been curtailed by the high frequency of antimicrobial resistance (1). Tetracycline

resistance genes are usually mobile plasmid or transposons encoded and thus resistance arise due to acquisition of new genes from external sources (2). Resistance is primarily due to either energy-dependent efflux of tetracycline or protection of the ribosomes from the action of tetracycline. Efflux as a means of antibiotic resistance is most commonly associated with tetracyclines (e.g. *tet*A, *tet*B, *tet*K pumps) (3). In clinical settings, exposure

to antibiotics promotes the mutational overexpression of active or silent multidrug transporters, leading to increased antibiotic resistance without acquisition of multiple, specific resistance determinants. Efflux pump genes such as *tet*A (B) of transposon Tn10 are induced by tetracyclines. This regulation is mediated by a repressor protein which prevents expression of *tet*A (B) when cells grow in the absence of tetracycline. When tetracycline is present, it enters the cell and binds the repressor and activates it, resulting in induction of the pump (4-9). The paradoxical ability of multidrug transporters to recognize and efficiently expel from cells scores of dissimilar organic compounds has been in the focus of extensive research for many years (10,11). Although the functions of drug transporters may involve both the protection of bacteria from outside toxins and the transport of natural metabolites, their clinical importance lies largely in providing Gram-positive pathogens with resistance to macrolides, tetracyclines and fluoroquinolones (12). The

enteric bacteria exhibit mostly complex multidrug resistance systems which are often regulated by operons or regulons (13). Tetracycline resistance could be encoded by *tet*A, *tet*B, or *tet*C (14).

Therefore, knowledge on the molecular mechanisms underlying antibiotic resistance will be of help for controlling multidrug-resistant (MDR) bacteria. We therefore discerned for the genes present in phenotypically tetracycline resistant *Salmonella enterica* serovar *typhimurium, typhi and enteritidis* strains isolated from diarrheal patients in Western Kenya.

PATIENTS and METHODS

Bacterial strains, growth conditions, and resistance determinations: As part of the ongoing project to identify the mechanisms and sources of antimicrobial resistance genes, *Salmonella* isolates obtained from diarrheal patients in the previous study by Onyango *et al*., (15) were used for this experiment. The resistance phenotypes were then determined accordingly (16).

Table 1. PCR primers targeting *tet* efflux pump in *Salmonella* isolates

Primer pair	Class target	sequence	PCR annealing and extension temperature (°C)[¥]	Amplicon size (bp)	References
*tet*A –FW	A	5′- CCTACATCCTGCTTGCCTTC-3′			
*tet*A –RV		3′ - CATAGATCGCCGTGAAGAGG-5′	53	210	17
*tet*AC –FW	A	5′-CGCYTATATYGCCGAAYATCAC-3′			
*tet*AC –RV	C	5′-CCRAAAWKCGGCWAGCGA - 3′	55	417	18
*tet*B –FW	B	5′-TTGGTTAGGGGCAAGTTTTG- 3′			
*tet*B –RV		3′ - GTAATGGGCCAATAACACCG-5′	53	659	17
*tet*BDEFHJ-FW	B	5′- GGDATTGGBCTTATYATGCC-3′,			
*tet*BD-RV		5′-ATMACKCCCTGYAATGCA -3′	55	967	18
*tet*G – FW	G	5′- CAGCTTTCGGATTCTTACGG- 3′			18
*tet*G – RV		3′– GATTGGTGAGGCTCGTTAGC-5′	53	844	17
*tet*GY – FW	G	3′- TATGCRTTKATGCAGGTC-5′			
*tet*Y – RV		5′- GACRAKCCAAACCCAACC-3′	55	917	18
*tet*EJH – RV	E,H,J	5′- AWDGTGGCDGGAATTTG -3′	55	650	18

[¥] Hot start PCR

Table 2. *tet* efflux detected in total *Salmonella* species chromosomal DNA from human stool

Bacteria strain	*tet* gene						
	*tet*A	*tet*B	*tet*C	*tet*D	*tet*G	*tet*(E,H,J)	*tet*AC
S. typhi	+(1)	+(3)	-(0)	+(3)	+(3)	+(5)	+(3)
S.typhimurium	+(5)	-(0)	-(0)	-(0)	-(0)	-(0)	-(0)
S. enteritidis	+(2)	+(1)	+(1)	-(0)	-(0)	-(0)	-(0)

(-): *tet* gene absent, (+): *tet* gene present

Molecular determination of tetracycline efflux pump: A total of 45 epidemiologically unrelated tetracycline-resistant isolates of 21 *Salmonella enterica* serovar *Typhimurium*, 4 *Enteritidis* and 17 *Typhi* and indeterminate 3 *Salmonella*, isolated from humans were investigated for the presence of tetracycline resistance genes. The detection of *tet* genes was performed by specific PCR amplification assays accordingly (17,18) (table 1).

RESULTS

Phenotypic tetracycline resistance analysis: All three *Salmonella* strains that were phenotypically resistant to tetracycline were tested for tetracycline resistance gene. (figures1 and 2).

Genotypic tetracycline efflux pump analysis: Tetracycline resistance genes (*tet* A, B, C, D, G, E, H, J, and AC) were isolated in 62.2% (n=28) *Salmonella* isolates. Of these, 64.3% (n=18/28) were *S. typhi*, 17.8% (n=5/28) *S. typhimurium*, and 14.3% (n=4/28) were *S. enteritidis*. *S. typhi*

expressed resistance genes to *tet*A, *tet*B, *tet*D, *tet*G, *tet*E,H,J, and *tet*AC in 3.6% (n=1/28), 10.7% (n=3/28), 10.7% (n=3/28), 10.7% (n=3/28), 10.7% (n=3/28) and 17.8% (n=5/28), respectively, in isolation or in combination. All 5 (17.8%) *S. typhimurium* partly expressed *tet*A of 210bp (figure 3) and *tet*AC of 417bp gene (figure 4).

Totally, 2 (7.1%) *S. enteritidis* displayed *tet*A resistance, however, *tet*B and *tet*C were found in 1 (3.6%) each (table 2). Three *S. typhi* isolates displayed either in isolation or combination of *tet* B, D, G, E, H, J, and AC (figure 4) of 659 and 917bp respectively; while one *S. enteritidis* isolate displayed *tet*A, and *tet*C.

DISCUSSION

Although TC can diffuse readily through the IM bilayer (19,20) the lipid bilayer of the outer membrane (OM) is relatively impermeable to lipophilic solutes (21) such as TC, and TC is thought to cross the OM mainly via the porin
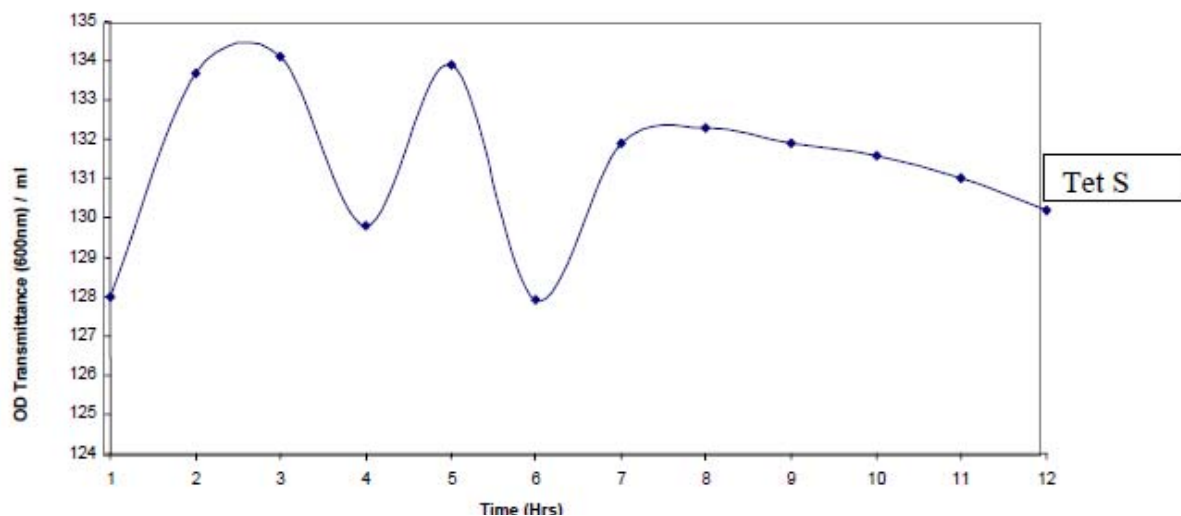

Figure 1. Tetracycline sensitive *S. typhimurium* spectrophotometry optical density (OD $_{600}$=2.0 – 2.10) assay curve. There was exponential growth of bacteria population within the first one and a half hours, and then the population declined for the same period of time after a while. The bacteria growth picked up for two hours and then declined for an hour and then picked up again for two hours. The population then constantly declined to zero. When this was looked to in light of normal bacteria sigmoid growth curve in a batch culture system, it was realized that during the stationary phase (after 3 hours) the smooth bacteria plateau was not observed, instead a smooth wavy curve with constant amplitudes was observed. Then followed a significant decline of bacteria population after eight hours from the start with a constant wavy drop to zero. Active efflux of tetracycline (TC) is observed as resistance mechanism. The *tet* protein can be observed to bind TC in the cytoplasm and pumps it, possibly into the periplasm, in exchange for a proton, a process that is driven by the proton motive force across the inner membrane (IM) as depicted by the curve in the first six hours of growth in this study.
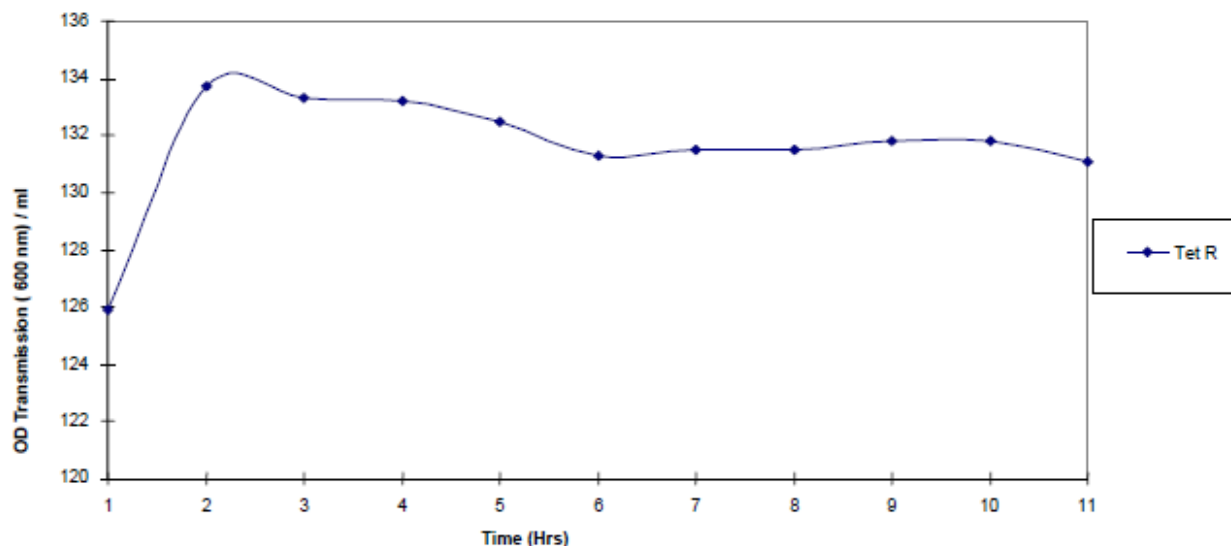
Figure 2. Tetracycline resistant *S. typhimurium* spectrophotometry optical density (OD 600=2.0 – 2.10) assay curve. There was exponential growth of bacteria population within the first two hours, and then the population declined slightly for half an hour, then plateaued for an hour and then declined constantly for two hours. The bacteria population then maintained a constant plateau to infinity. Looking at this in the light of normal bacteria sigmoid growth curve in a batch culture system, it was realized that during the stationary phase (after 2 hours) the smooth bacteria growth curve plateau was observed for three hours. These bacteria population was then maintained to infinity
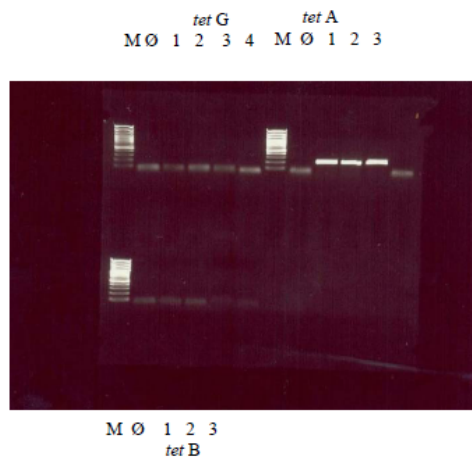

Figure 3. PCR gel bands showing *tet*G, *tet*A, and *tet*B in *S. typhimurium*. The bands indicated the molecular weight of the genes in base pairs (M=molecular DNA maker 100bp, ø=negative control, and numerical numbers the tested isolates.
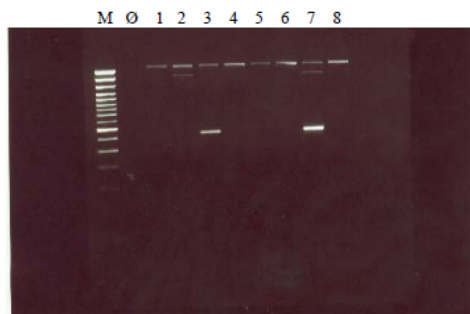

Figure 4. PCR gel bands showing *tet*AC in *S. typhmuriumi*. The bands indicated the molecular weight of the genes in base pairs (M=molecular DNA maker 100bp, ø=negative control, and numerical numbers the tested isolates.

OmpF (22). This observation was not as achieved in this setup since it is thought that both TC entered and exits the cell through OmpF. Expected decreased OmpF levels should affect both the influx and efflux of TC equally and that the steady-state level of TC inside the cell would remain unchanged as would be expected for a tetracycline sensitive isolate. In addition, it is not clear how pumping of TC into the periplasm by *tet* leads to high levels of resistance, since TC should easily be able to reenter the cytoplasm by spontaneous diffusion.

Since high resistance may not occur as a result of multidrug-resistant (MDR) efflux pumps alone, the association of over-expression of these genes amongst highly resistant clinical isolates cannot be ignored (23). Tetracycline resistance was mainly mediated by *tet*AC with a few isolates displaying *tet*A, B, D, G, E, H, and J, in equal proportions in this study. *tet*A is located frequently on transposons such as Tn1721, and the gene has been widespread among Gram-negative bacteria including *Salmonella* (24,25). The amplification results of *tet*A gene indicated the presence of *tet*A gene mediating a proton–pump protein. This observation was also made by Gebreyes and Altiers (17). The pump operates under the influence of electromotive force and hence energy dependent. This observation could fit well with the wavy curvatures observed by spectrophotometer where, there was drug trafficking into the bacteria cell and then after a while, it was pumped out of the cell through the antiport membrane protein porins. Surprisingly, only one of the *tet*A resistant *S. typhimurium* isolate would have its *tet*A gene on plasmid with a 0.8Kb integron. The other four had the *tet*A resistance gene with no integron (27) implying that resistance was chromosomally coded. This mechanism of resistance by chromosomal genes is activated by induction or mutation caused by the stress of exposure to antibiotics in natural and clinical environments (13,26). The display of *tet* gene in combination in *S. typhi* and *S. enteritidis*

observed in this study was an indication of existence of these resistance genes in cluster of SGI 1 reported to contain two class 1 integrons, the chloramphenicol and florfenicol resistance gene, *flo*R, and the tetracycline resistance gene *tet* (G) (27). This phenomenon was also observed in previous studies by Onyango et al (15).

Molecular observation of this study indicates clearly that the presence of tetracycline drug into the *S. typhimurium* bacteria cells lead to its binding to the repressor proteins and hence reducing the affinity of the repressor protein to the DNA regulator region. In this case, the transcription of the efflux proteins was enhanced leading to the extrusion of the tetracycline drug outside the periplasmic membranes. This lead to low tetracycline in the bacteria cell and triggered the binding of the repressor protein to the DNA regulator region. This phenomenon was repeated until there was no drug within the bacteria cell and hence the wavy spectrophotometric curves observed. Therefore, the tetracycline resistance genes were verified to confer the resistance due to the efflux of tetracycline from the cell catalyzed by drug: H^+ antiport (16,28).

In conclusion, we herein report the existence of chromosomally coded tetracycline efflux pump in the isolated *Salmonella* isolates from diarrheal patients in Western Kenya. However, the resistance pattern was very low compared to other regions.

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REFERENCES

1. Kariuki S, Mirza NB, Wasteson Y, Senerwa D, Gathuma JM, Orjan O. Tetracycline resistance genes in Kenya hospital isolates of *S. typhimurium*. APMIS. 1992;100:629-34.

2. Poole K. Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. J Mol Microbiol Biotechnol. 2001;3:255-64.

3. Poole K. Outer membranes and efflux: The path to multidrug resistance in gram-negative bacteria. Curr Pharm Biotechnol. 2002;3:77– 98.

4. Olga L, Wil W. Inhibition of efflux pumps as a novel approach to combat drug resistance in bacteria. J Mol Microbiol Biotechnol. 2001;3(2):225-36.

5. Levy SB, McMurry L. Detection of an inducible membrane protein associated with R factor mediated tetracycline resistance. Biochem Biophys Res Commun. 1994;56:1060-68.

6. Jorgensen RA, Reznikoff WS. Organization of structural and regulatory genes that mediate tetracycline resistance in transposon TnlO. J Bacteriol. 1979;138:705-14.

7. Jr Wray LV, Jorgensen RJ, Reznikoff WS. Identification of the tetracycline resistance promoter and repressor in transposon TnlO. J Bacteriol. 1981; 147:297-304.

8. Bertrand KP, Postle K, Jr Wray LV, Reznikoff WS. Overlapping divergent promoters control expression of Tn1O tetracycline resistance. Gene. 1983;23:149-56.

9. Hillen WK, Schollmeier K, Gatz C. Control of expression of the TnlO-encoded tetracycline resistance operon: interaction of RNA polymerase and TET repressor with the *tet* operon regulatory region. J Mol. Biol. 1984;172:185-201.

10. Raḿon-Garćia S, Carlos M, Jośe AA, Edda R. Characterization of tetracycline resistance mediated by the efflux pump Tap from *Mycobacterium fortuitum*. J Antimicrob Chemother. 2006; 57:252-59.

11. Hellen IZ. Molecular analysis of efflux pump-based antibiotic resistance. Int J Med Microbiol. 2004;292(2): 95-105.

12. Penelope NM, Alex AN. Efflux–mediated drug resistance in Gram-positive bacteria. Curr Opinion Microbiol. 2001;4(5):509–14.

13. Antony MG. Multi-drug resistance in enteric and other Gram-negative bacteria. FEMS Microbiol Letters. 1996;139(1):1-10.

14. Faldynova M, Pravcova M, Sisak F, Harlickova H, Kolackova I, Cizek A, et al. Evolution of antibiotic resistance in the Czech Republic between 1984 and 2002. Antimicrobiol Agents Chemother. 2003;47(6): 2002–5.

15. Onyango DM, Sarah W, Rose K, Eliud NW. Isolation of Salmonella and Shigella from fish harvested from Winam Gulf of Lake Victoria, Kenya. J Infect Developing Countries. 2009;3(1):36-41.

16. Paulsen IT, Brown MH, Skurray RA. Proton–dependent multidrug efflux systems. Microbiol Rev. 1996;60:575-608

17. Gebreyes WA, Altier C. Molecular characterization of multidrug–resistant *S. enterica* subsp. enterica serovar typhimurium isolates from swine. J Clin Microbiol. 2002;40(8):2813–22.

18. Furushita M, Shiba T, Maeda T, Yahata M, Kaneoka A, Takahashi Y, et al. Similarity of tetracycline resistance genes isolated from fish farm bacteria to those from clinical isolates. Appl Environ Microbiol. 2003;69: 5336–42.

19. Argast M, Beck CF. Tetracycline uptake by susceptible Escherichia coli cells. Arch Microbiol. 1985;141:260–65.

20. Nikaido H, Thanassi DC. Penetration of lipophilic agents with multiple protonation sites into bacterial cells: tetracyclines and fluoroquinolones as examples. Antimicrob Agents Chemother. 1993;37:1393–99.

21. Plesiat P, Nikaido H. Outer membranes of Gram-negative bacteria are permeable to steroid probes. Mol Microbiol. 1992;6:1323–33.

22. Chopra I, Hawkey PM, Hinton M. Tetracyclines, molecular and clinical aspects. J Antimicrob Chemother. 1992;29:245–77.

23. Webber MA, Piddock LJ. The importance of efflux pumps in bacterial antibiotic resistance. J Antimicrobiol Chemother. 2003;51:9-11.

24. Aarestrup MF, Monton L, Mary CE, Aroon B, Thongchai C, Rene S, et al. Antimicrobial susceptibility and occurrence of resistance genes among *Salmonella enterica* Weltevreden from different countries. 2003;52: 715–18.

25. Frech G, Schwarz S. Molecular analysis of tetracycline resistance in *S. enterica* susp. enterica serovars typhimurium, enteritidis, Dublin, Cholerasuis, Hadar and Saintpaul: construction and application of specific gene probes. J Appl Microbiol. 2000;89:633–41.

26. Indalo AA. Antibiotic sale behavior in Nairobi: a contributing factor to antimicrobial drug resistance. East Afr Med J. 1997;74:171–73.

27. Ashraf MA, Kimi F, Kei S, Hidekazu K, Tadashi S. Characterization of multidrug–resistant isolates of *Salmonella paratyphi* B from Japan. J Antimicrobiol Chemother. 2005;56:250–54.

28. Onyango DM. Drug susceptibilities and mechanisms of drug resistance among *Salmonella typhimurium* isolates from persons in Western Kenya. PhD thesis. Maseno University, 2007.