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Construction and Testing of EGFP Based Bacterial Biosensor for the Detection of Residual Tetracyclines in Milk and Water

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Abstract: A plasmid containing a transcriptional fusion between *tetR* regulated *tet* promoter from plasmid pOT182 and Enhanced Green Fluorescent Protein (EGFP) gene was created and was transformed into *E. coli* JM109 and this strain was used as whole cell bacterial biosensor for detection of tetracyclines in milk and water samples. The sensor strain *E. coli* JM109 (pJSKV41) was able to detect tetracycline in the range of 10-60 ng mL^{-1} sample and oxytetracycline in the range of 25-125 ng mL^{-1} of sample. When employed for detecting residual tetracyclines in pond water samples, the biosensor strain showed high sensitivity. Also the biosensor strain was able to detect residual tetracycline in goat milk even after 4 days of tetracycline treatment.

Key words: Antibiotic resistance, biosensor, EGFP, tetracycline detection

INTRODUCTION

In the recent past, there has been a dramatic increase in the prevalence of multidrug resistant human pathogenic bacteria, resulting in an escalation of mortality worldwide due to infection caused by them (Aubel *et al*., 2001). A major factor contributing to this increased drug resistance among pathogenic microbes is the unscrupulous use of antibiotics for medical, veterinary and agricultural purposes (Levy, 2002). In Europe about 10,000 t of antibiotics are consumed per year and it has been estimated that of the total quantity of antibiotics consumed, a high proportion is released to environment through various discharges (Kummerer *et al*., 2000). Antibiotics such as tetracyclines or quinolones have half-lives up to several hundred days in aquatic environments (Kummerer, 2001). This has caused a sustained presence of residual antimicrobial agents in environment. Antibiotic contamination of water also perturb microbial ecology, present challenges for water industry on the issues of water reuse and water resource planning. As the frequency of tetracycline resistant pathogens are very high, medical uses of tetracyclines have diminished considerably, but they are still heavily used in veterinary and agricultural purposes.

The prophylactic and subtherapeutic level usage of tetracyclines for veterinary purposes has also resulted in the presence of residual tetracyclines in biological samples like milk. This scenario calls for continuous monitoring of water samples and biological samples like milk for the presence of residual tetracyclines and will require highly sensitive, easy to use continuous detection systems. Several methods like HPLC and immuno-detection are available for the detection of antibiotics in various samples (Kumar *et al*., 2004). However, these methods are expensive and require highly trained personnel for testing. FDA approved and widely used microbial inhibition tests like *Bacillus stearothermophilus* Disc Assay (BSDA) for detecting antibiotic residues in milk, is not specific for antibiotics like tetracyclines.

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With the advances in genetic engineering, use of recombinant whole cell bacterial biosensors for the detection of antibiotics has been indicated by Bahl *et al*. (2004), Hansen and Sorensen (2000) and Hansen *et al*. (2001). A major advantage of using biosensor bacteria to detect or quantify specific compounds in natural samples is that only the bioavailable fraction is detected. Hence, the measurements made are relevant to the effects of compounds on microbial community. Of the several reporter genes that are available for the development of bacterial biosensors, use of GFP as reporter is preferable as it does not require any cofactors for expression and can be detected easily without cell lysis or addition of any reagents. Earlier studies in our lab have shown that use of novel GFP variants like Enhanced Green Fluorescent protein (EGFP) gives better signals than wild type GFP when used as reporter gene in bacterial biosensor for arsenic (data not shown). In this report we present the development and evaluation of an EGFP based recombinant *E. coli* strain for the detection of tetracyclines in milk and water samples.

MATERIALS AND METHODS

Host Strain, Plasmids and Reagent

E. coli JM109 was used aas the host for recombinant DNA protocols and biosensor assays. Axenic culture of *E. coli* JM109 was growing either in Luria Bertani (LB) both or LB agar purchased from Himedia, India. The Tn5 transposon containing plasmid pOT182 used as the template for PCR was selected based on searches in online database ARGO and was obtained from National Institute of Genetics, Mishima Japan. All the enzymes used for DNA manipulations, PCR and cloning were Purchased from New England Biolabs (USA).

Fluorescence Microscopy

EGFP expression in recombinant bacteria was analyzed and documented with an Olympus CKX41 epifluorescence microscope fitted with an Olympus Comedia C400 digital camera. Bacterial cells was viwed spread on a gelatin coated glass slide and were viwed by exciting with a 100 W mercury arc lamp and filter block fitted with a 465-495 nm exciting filter and a 500-540 nm emission filter. Photomicrograph were captured with 11 X digital zoom, ISO200 light sensitivity and automatic capture moad.

Fluorescence Assay

EGFP expression levels in Biosensor bacterial cells were estimated with a Wallac Victor Multilabel counter. All instrumental conditions were optimized for maximum sensitivity as described by the manufacturer. To minimize the background fluorescence, low background 96 well plates (Tarsons, Mumbai) were used. For measurements, 200 μL of bacterial suspension was pipetted into each well and fluorescence was estimated by exciting the cells at 580 nm and emission was detected using a 510 nm filter set. All measurements were performed in triplicates.

Biosensor Strain Construction

All procedures for DNA manipulations, cloning and PCR were carried out according to standard protocols (Sambrook *et al*., 1989). For the construction of tetracycline regulated biosensor strain, we have used the tetracycline regulatory elements in plasmid pOT182 (Merriman and Lamont, 1993). The *tetR, tet* promoter and part of *tetA* sequence were amplified using PCR from plasmid pOT182 using oligonucleotide primers incorporating *Hind*III and *Sal*I restriction endonuclease sites (restriction sites in bold, 5'-GCGAAGCTTGTTGTCTACATGGCTCTGCTG-3', 5'-GCGTCGACCTCAAATTACGATCAGGGGTAT-3'). The primers were designed based on the published sequence of plasmid pOT182 (Genbank accession No. U73849). The resulting 856 bp fragment was restriction digested with *Hind*III and *Sal*I and ligated with T4 DNA ligase into plasmid pEGFP (Clontech, USA) cut with same set of enzymes. Recombinants were selected by randomly

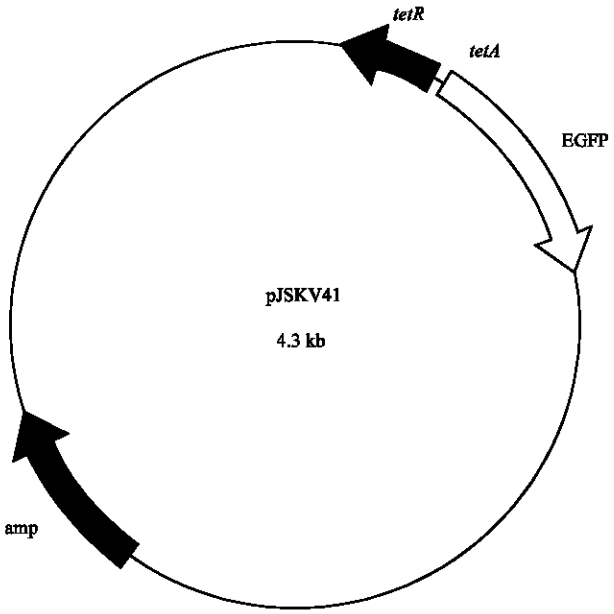

Fig. 1: Structure of pJSKV41: *tetR*, *tet* promoter and part of *tetA* are cloned upstream of the reporter gene EGFP

picking the colonies from transformation plates containing 100 μg mL^{-1} ampicillin and 50 ng mL^{-1} tetracycline and viewing the cells under an Olympus CKX41 epifluorescence microscope. Colonies exhibiting green fluorescence were selected for further analysis and the presence of insert with correct orientation was confirmed by double digestion with *Hind*III and *Sal*I. The resulting clone was designated as pJSKV41 (Fig. 1) and was transformed into competent cells of *E. coli* JM109 using conventional protocols.

Estimation of Tetracyclines in Water

Single colonies of *E. coli* JM109(pJSKV41) were picked from Luria Bertani (LB) agar medium containing 100 μg mL^{-1} of ampicillin and was grown for 10 h in LB broth containing appropriate amount of ampicillin. After inoculating 1% of this original cell suspension into fresh LB medium in a sterile 50 mL test tube and was incubated at 37°C with vigorous shaking until OD_{600} reached 0.9. Optimal incubation time required for the biosensor assay was determined by mixing 2.5 mL of this cell suspension and 2.5 mL of de-ionized water containing 50 ng mL^{-1} tetracycline in sterile 50 mL test tube and this was incubated at 37°C with 150 rpm shaking. Tetracycline induced EGFP in the biosensor cells was estimated at regular intervals by aseptically pipetting out 200 μLof this cell suspension into a low background fluorescence microtiter plate and fluorescence was measured with a Wallac 1420 Victor3^{TM} multilabel counter (Wallac Oy, Turku, Finland). For generating a dose response curve, 2.5 mL of deionized water with varying concentrations of tetracycline or oxytetracycline and 2.5 mL of biosensor cell suspension were mixed in sterile test tubes and were incubated at 37 °C for 12 h with 150 rpm shaking. After incubation, the fluorescence from the cells was estimated using multilabel counter as described above. For assessing the efficacy of the strain in detecting residual tetracyclines in environmental water samples, the strain was tested against tetracycline spiked pond water samples. To make the water samples free of microbes, collected pond water (pH 7.4) was filtered through a 0.22 μm membrane filter and was spiked with varying

concentrations of tetracycline (10, 30 and 60 ng mL^{-1}) and oxytetracycline (25, 75 and 125 ng mL^{-1}). 2.5 mL of these samples and 2.5 mL of biosensor cells grown as described earlier was mixed and incubated at 37°C for 12 h and fluorescence levels were determined in similar conditions as mentioned before.

Estimation of Tetracyclines in Milk and Comparison with BSDA Method

The efficacy of biosensor in detecting tetracyclines in biological samples was determined by using this strain to detect residual tetracyclines in milk samples. To obtain tetracycline containing milk samples, a lactating goat producing 1.0 L day^{-1} milk and weighing 15 kg was given a single intravenous dose of 40 mg kg^{-1} tetracycline and milk samples were collected at every 12 h interval after the injection. After three weeks period, the same goat was given a dose of 30 mg kg^{-1} oxytetracycline and milk samples were collected in the same manner. Another lactating goat of the same size served as the control. Tetracycline treated goat milk and control milk from the control goat was centrifuged at 12000 rpm for 10 min and supernatant was filtered through a 0.22 μm membrane filter. 2.5 mL of diluted milk filtrate and 2.5 mL of biosensor cell suspension grown as described previously was mixed in a 50 mL culture tube and was incubated at 37°C for 12 h with 150 rpm shaking and fluorescence levels were estimated as mentioned previously. The concentrations of tetracycline or oxytetracycline in the samples were calculated by extrapolating the values against standard curves. For comparing the efficacy of biosensor with the widely used BSDA method, axenic culture of *B. stearothermophilus* was obtained from National Collection of Industrial Microorganisms (NCIM) Pune, India and the assay was performed as per standard protocols (AOAC, 1984).

RESLUTS AND DISCUSSION

Continuous release of residual antibiotics like tetracycline into the environment is a matter of concern as it promotes the proliferation of multidrug resistant bacteria. Here, we report the development of an *E. coli* strain for the detection of tetracyclines in environmental samples. In the biosensor strain developed, we have coupled 856 bp region of *tet* operon, containing *tetR* gene, *tet* promoter and part of *tetA* to the reporter gene EGFP, so that the EGFP expression can be used to estimate the amount of tetracycline that induces the operon. The recombinant plasmid pJSKV41 was transformed into *E. coli* JM109 and the recombinants selected from LB agar plates containing 50 ng mL^{-1} tetracycline exhibited bright fluorescence when observed under an epifluorescence microscope. EGFP is a red shifted variant of GFP with higher extinction coefficient and brighter fluorescence than wild type GFP and retains its activity after N-terminal fusions (Patterson *et al*., 1997). In order to determine the optimum time required for the detection of tetracycline induced EGFP expression in biosensor cells, an induction time course in the presence of 50 ng mL^{-1} tetracycline was performed. The results indicate that although EGFP expression could be seen as early as six hours after incubation with tetracycline, significant fluorescence levels above background was observed beyond 12 h (Fig. 2). Hence, this period was taken as the optimum time required for all further measurements.

A dose response curve of the EGFP expression in biosensor cells against tetracycline and its derivative oxytetracycline was generated by incubating the biosensor cells with varying concentrations of these antibiotics. The detection range of tetracycline was between 10-60 ng mL^{-1} (Fig. 3) whereas for oxytetracycline the range was between 25-125 ng mL^{-1} (Fig. 4). Antibacterial action of tetracycline is produced by the inhibition of protein synthesis at ribosomal level. Tetracycline at higher concentrations inhibits the expression of EGFP in biosensor cells. However, the affinity of tetracyclines is 1000 times higher to *tetR* repressor protein than to ribosomes and low concentrations of tetracyclines activate GFP expression, but since the functional *tetA* is absent in the biosensor cells, at higher concentrations the protein synthesis as well GFP expression is inhibited (Hansen and

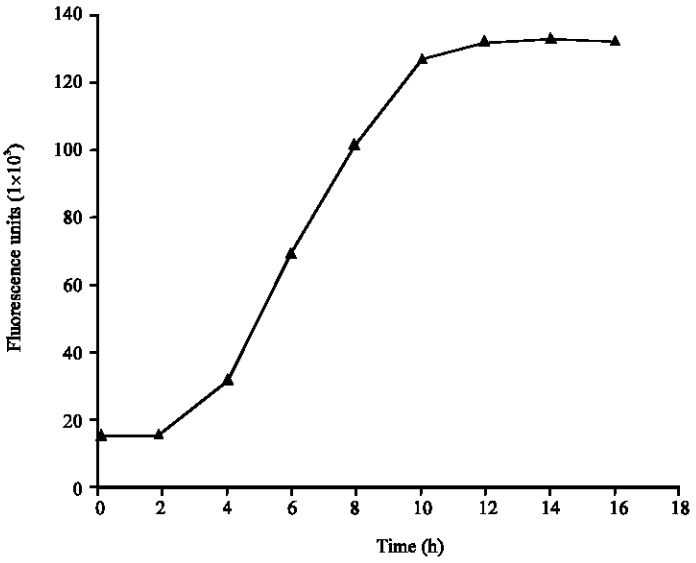

Fig. 2: Time course induction of EGFP by tetracycline. 2.5 mL water containing 50 ng mL^{-1} of tetracycline and 2.5 mL biosensor sensor cells (OD_{600} = 0.9) were mixed and was incubated at 37°C and fluorescence levels were measured by a multilabel counter at every 2 h. Background fluorescence values have been subtracted from each value and each point represented is average of three replicates

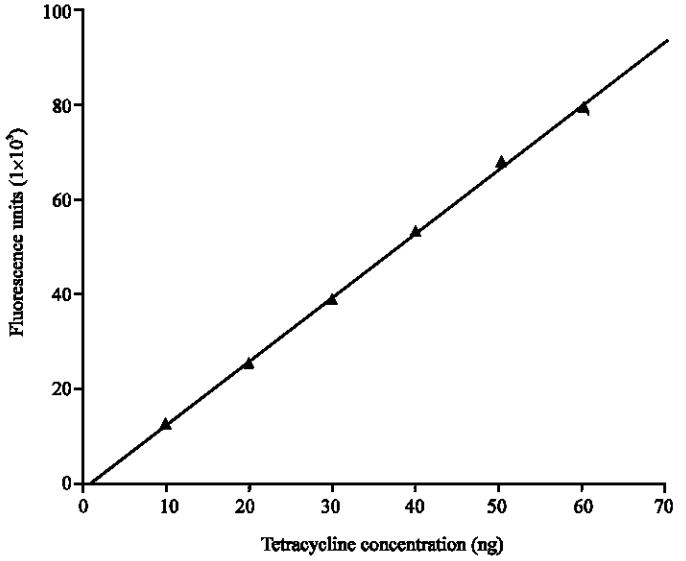

Fig. 3: Induction in biosensor cells against known concentrations of tetracycline. 2.5 mL de-ionized water containing varying amount of tetracycline (10- 60 ng mL^{-1}) and 2.5 mL biosensor sensor cells (OD_{600} = 0.9) were mixed and was incubated for 12 h at 37°C and fluorescence levels were measured by a multilabel counter. Background fluorescence values have been subtracted from each value and each point represented is average of three replicates

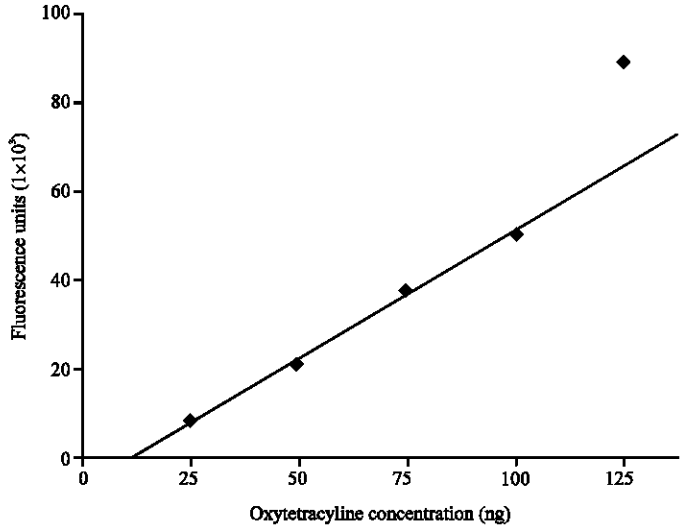

Fig. 4: Induction in biosensor cells against known concentrations of oxytetracycline. 2.5 mL de-ionized water containing varying amount of oxytetracycline (25-125 ng mL^{-1}) and 2.5 mL biosensor sensor cells (OD_{600} = 0.9) were mixed and was incubated for 12 h at 37°C and fluorescence levels were measured by a multilabel counter. Background fluorescence values have been subtracted from each value and each point represented is average of three replicates

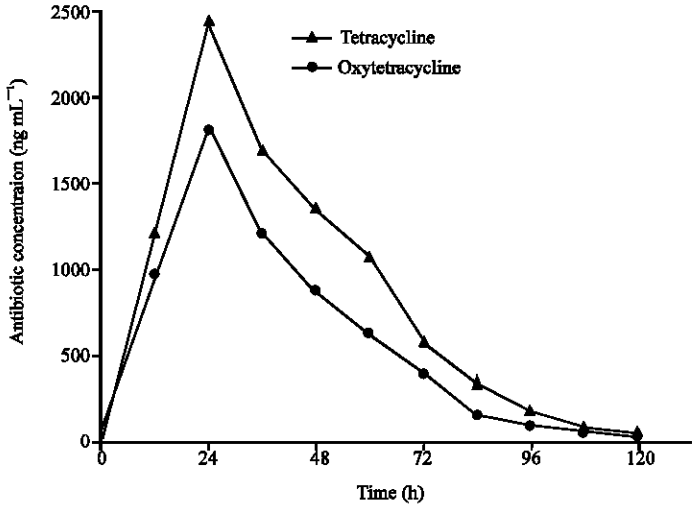

Fig. 5: Detection of tetracycline and oxytetracycline in milk samples. A healthy goat given a single intravenous injection of 40 mg kg^{-1} tetracycline or 30 mg kg^{-1} oxytetracycline and milk was collected before injection and at every 12 h after injection. Milk was filtered through a 0.22 μm membrane filter and 2.5 mL of diluted milk filtrate and 2.5 mL of biosensor sensor cells (OD_{600} = 0.9) and was incubated for 12 h at 37°C and fluorescence levels were measured by a multilabel counter. Each point represented is average of three replicates

Sorensen, 2000; Hansen *et al*., 2001). Hence, the biosensor responds to sublethal concentrations of tetracyclines in a concentration dependent manner.

The detection range of the biosensor also is greatly determined by the type of reporter gene used and also by the sensitivity of instruments used to detect reporter gene expression. Studies have shown that when wild type GFP was used as the reporter gene, it did not produce sufficient fluorescence even by Fluorescence Activated Cell Sorter (FACS). Use of different GFP variants improves the detection capacity and when FACS optimized GFP variant was used the fluorescence levels were 21 times higher than that of wild type GFP (Hansen *et al*., 2001). In this study we have used a different GFP variant (EGFP) which is 36 times more fluorescent than wild type GFP (Cormack *et al*., 1996) and this will help to detect the GFP expression in biosensor cells with less sensitive instruments than FACS and hence can bring down the cost of instruments required for GFP detection.

We also evaluated the efficiency of the biosensor strain in detecting tetracyclines in biological samples like milk and compared the results with widely used BSDA method. Milk samples were collected from a goat given tetracycline or oxytetracycline injection and the antibiotic concentration in the samples was measured by biosensor cells. Figure 5 show that highest amount of antibiotic was found after 24 h of antibiotic injection and up to five days detectable amount of antibiotic persisted in milk. When the same samples were assayed with BSDA, the assay detected residual antibiotic up to 86 h (zone of inhibition >16 mm) whereas biosensor assay showed the presence of tetracyclines up to 120 h. BSDA is an FDA approved protocol for screening milk samples for the presence of antibiotics and is extremely sensitive for detecting antibiotics like β-lactams. However, it does not detect tetracyclines below 200 ng mL^{-1} of milk. The maximum residue limit specified by European Union (EU) for tetracyclines in milk samples in 100 ng mL^{-1} and hence BSDA is not sensitive enough to comply with such standards (Nouws *et al*., 1998). Further, BSDA is a qualitative test rather than a quantitative one. In contrast, biosensor cells detected both tetracycline and oxytetracycline in a quantitative manner well below EU specifications. Also the detection range of biosensor cells for tetracycline and oxytetracycline is overlapping and hence the developed strain could be used to detect tetracycline and its common derivatives.

Presence of residual tetracyclines in milk is a common occurrence as it is frequently used to treat cattle diseases like mastitis (Sudershan and Bhat, 1995; Chopra and Roberts, 2001). This affects the quality of milk, contaminates other milk lots when such samples are mixed and could result in passive intake of tetracyclines if these samples are used for human consumption. Monitoring of tetracycline residues in dairy industry will require testing of large number of samples on a daily basis. As easy to use testing protocols like BSDA is not sensitive enough for antibiotics like tetracyclines, cheaper alternatives are required. Reporter gene like GFP require only simple excitation with UV light and detection could be carried out by multilabel counter like Wallac Victor which supports high throughput processing of samples. Hence, the biosensor strain developed in this study could also be used in dairy industry for screening milk samples.

CONCLUSION

The bacterial biosensor developed in this study has potential applications in the detection of residual tetracycline and oxytetracylcines in water and milk samples. The use of high throughput analysis also makes quantitative measurements with high accuracy.

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REFERENCES

AOAC, 1984. Official Methods of Analysis. 14th Edn., Association of Official Analytical Chemists, Washington, DC. USA., ISBN: 0-935584-24-2.

Aubel, D., R. Morris, B. Lennon, M. Rimann, H. Kautmann, M. Folchet, J.E. Bailey, C.J. Thompson and M. Fussenegger, 2001. Design of novel mammalian screening system for the detection of bioavailable, non-cytotoxic streptogamin antibiotics. J. Antibiot., 54: 44-55.

Bahl, M.I., L.H. Hansen, T.R. Licht and S.J. Sorensen, 2004. *In vivo* detection and quantification of tetracycline by use of a whole cell biosensor in rat intestine. Antimicro. Agent Chemother., 48: 1112-1117.

Chopra, I. and M. Robert, 2001. Tetracycline antibiotics: Mode of action, applications, molecular biology and epidemiology of bacterial resistance. Microbiol. Mol. Biol. Rev., 65: 232-260.

Cormack, B.P., R.H. Valdivia and S. Falkow, 1996. FACS-optimized mutants of the green fluorescent protein (GFP). Gene, 173: 33-38.

Hansen, L.H. and S.J. Sorensen, 2000. Detection and quantification of tetracyclines by whole cell biosensors. FEMS. Microbiol. Lett., 190: 273-278.

Hansen, L.H., B. Ferrari, A.H. Sorensen, H. Veal and S.J. Sorensen, 2001. Detection of oxytetracycline production by streptomyces rimosus in soil microcosoms by combining whole cell biosensors and flow cytometry. Applied Environ. Microbiol., 67: 239-244.

Kumar, K., A. Thompson, A.K. Singh, Y. Chande and S.C. Gupta, 2004. Enzyme linked immunosorbent assay for ultra trace determination of antibiotics in aqueous samples. J. Environ. Qual., 33: 250-256.

Kummerer, K., Al-Ahmed and V. Mersch-Sundermann, 2000. Biodegradability of some antibiotics, elimination of the genotoxicity and affection of wastewater bacteria in a simple test. Chemosphere, 40: 701-710.

Kummerer, K., 2001. Drugs in the environment: Emission of drugs, diagnostic aids and disinfectants into wastewater by hospitals in relation to other sources. Chemosphere, 45: 957-969.

Levy, S.B., 2002. Factors impacting on the problem of antibiotic resistance. J. Antimicrob. Chemother., 49: 25-30.

Merriman, T.R. and I.L. Lamont, 1993. Construction of a self-cloning promoter probe vector for gram-negative bacteria. Gene, 126: 17-23.

Nouws, J., G. Loeffen, J. Schouten, H. van Egmond, H. Keukens and H. Stegeman, 1998. Testing of raw milk for tetracycline residues. J. Dairy Sci., 81: 2341-2345.

Patterson, G.H., S.M. Knobel, W.D. Sharif, S.R. Kain and D.W. Piston, 1997. Use of the green fluorescent protein and its mutants in quantitative fluorescence microscopy. J. Biophys., 73: 2782-2790.

Sambrook, J., E. Fritsch and T. Maniatis, 1989. Molecular Cloning: A laboratory Manual. Cold Spring Harbour Laboratory Press, New York.

Sudershan, R.V. and R.V. Bhat, 1995. A survey on veterinary drug use and residues in milk in Hyderabad. Food Addit. Contamin., 12: 645-650.