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PCR Analysis of Resistant Bacteria Strains Isolated from River Sokoto, Northwestern Nigeria

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ABSTRACT: Thirteen resistant bacteria strains from River Sokoto namely *Salmonella typhi*, *Escherichia coli*, *Shigella flexneri*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Bacillus subtilis*, *Enterobacter cloacae*, *Klebsiella oxytoca*, *Staphylococcus saprophyticus*, *Providencia rettgeri*, *Klebsiella pneumonae* and *Enterobacter aerogenes* recovered on Mueller-Hinton agar by disc diffusion method were subjected to PCR (Polymerase chain reaction) analysis to determine their antibiotic resistance genes. Forward and reverse copies of five primers (*TEM*, *spvC*, *SHV*, *aacC3* and *qnrS*) were used in the PCR analysis. Aminoglycoside resistance genes (*aacC3*) were detected in the majority of the isolates such as *E. coli*, *Shigella flexneri*, *Enterobacter cloacae*, *Staphylococcus saprophytica* and *Enterobacter aerogenes* with plasmid numbers ranging from 1 to 4 and molecular weights ranging from 185 bp to >10,200 bp. Virulence resistance genes (*spvC*) were detected in *Salmonella typhi* on two plasmids with molecular weights of 571 bp and >10,200 bp while quinolones resistance genes were detected on plasmid numbers ranging from 1 to 3 and molecular weights ranging from 400 bp to 1000 bp in *Staphylococcus aureus*, *Streptococcus faecalis*, *Bacillus subtilis* and *Providencia rettgeri*. Three different resistance genes namely β-lactam (bla_{TEM}), virulence (*spvC*) and quinolones (*qnrS*) on 6 plasmids with molecular weights ranging from 428 bp to 1,200 bp were found in *Pseudomonas aeruginosa* while two resistance genes (*aacC3* and *qnrS*) on 2 plasmids with molecular weights of 185bp and 428bp were detected in *Klebsiella pneumoneae*. Non-specific resistance genes alongside with specific resistance genes were however detected in the majority of the isolates. The study revealed that the resistance genes exhibited by resistant bacteria isolates from River Sokoto were mainly virulence, aminoglycoside and quinolones resistant genes. The scientists are therefore challenged on the need for development of new antibiotics to combat the infections caused by these resistant strains.

Keywords: PCR Analysis, Resistant Bacteria, Antibiotic Resistance Genes, Non-Specific Resistance Genes

INTRODUCTION

Antibiotic resistance represents a global health problem, requiring better understanding of the ecology of antibiotic resistance genes (ARG), their selection and their spread in the environment (Marti *et al*., 2013). The overuse of antibiotics in human medicine and agriculture is a growing concern for public health. Overuse combined with inadequate wastewater treatment has led to the presence of antibiotic resistant bacteria and genes encoding antibiotic resistance in surface waters, river sediments, and the faeces of wild animals exposed to waste residuals. The multiple resistance of bacteria against antibiotics resulted from the fact that domestic and industrial waste were emitted into threw away without clarifying and so affect the underground water resources (Alzahrani and Gherbawy, 2011). The presence of antibiotic resistant bacteria in water sources throughout the world has been documented (Hamner, 2006; Baquero *et al*., 2008; Oyedeji, *et al*., 2011). Exposure to antibiotic resistant bacteria and the corresponding antibiotic resistance genes (ARG) in surface water can occur when the waters are used for drinking or recreation (Alzahrani and Gherbawy, 2011). Infection by antibiotic-resistant strains of pathogenic bacteria can have severe health implications for the sickened individual including more virulent strains and less treatment options (Barza and Travers, 2002).

In Sokoto, Nigeria, scarcely or seldom investigation was done on the assessment of pathogenic bacterial strains in River water using molecular techniques. In this study, we therefore examined genotypic characteristics of antibiotic resistant bacteria isolates recovered from six sampling points on River Sokoto.

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MATERIALS AND METHODS

Thirteen resistant bacteria strains from River Sokoto namely *Salmonella typhi*, *Escherichia coli*, *Shigella flexneri*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Bacillus subtilis*, *Enterobacter cloacae*, *Klebsiella oxytoca*, *Staphylococcus saprophyticus*, *Providencia rettgeri*, *Klebsiella pneumonae* and *Enterobacter aerogenes* confirmed by sensitivity test on Mueller-Hinton agar were subjected to PCR analysis to determine their antibiotic resistance genes. The molecular characterization of the plasmid genes in the resistant isolates was carried out at DNA Labs, Kaduna, Nigeria and it involved the following steps:

Bacterial cell preparation

Bacterial cells were prepared using the method described by Dubey (2009). Single colonies were picked from freshly streaked isolates on MacConkey plate and incubated into 5ml Luria Bertani (LB) broth medium and incubated overnight at 37^{o}C for 18 – 24 hours. Young cells were then harvested in eppendorff tube by centrifugation at 4^{o}C, 8000rpm (6800xg) in a refrigerated micro-centrifuge for 30 seconds. The supernatants were decanted and cells harvested.

DNA extraction

DNA extraction of the bacterial cells was carried out by using a DNA extraction kit (QIAamp DNA mini kit; Qiagen K.K., Tokyo, Japan). Heating block was first set at 60^{o}C before starting the extraction. Into a 2ml tube was added 200µl of the bacterial cells in liquid medium. Four hundred microliter (400µl) of lyses buffer and 10µl proteinase K were added to the sample and the tube was placed on heat block at 60^{o}C for minimum of 1 hour. Four hundred microliter (400 µl) of phenol chloroform (1:1) was added to the lysate and vortexed briefly. The mixture was spinned at 10000rpm for 10 minutes to separate the phases. The upper layer was carefully removed with a pipette without taking the white interphase which contained the DNA. For the second time, 400µl of chloroform was added to the remainder and vortexed briefly. The mixture was spinned again at 10000rpm for 10 minutes to separate the phases and the upper layer was carefully removed without taking the white interphase. To the remainder, was added 400µl of 100% ethanol and 20µl of 3M sodium acetate. This was mixed by inverting the tube several times and the tube was incubated at –20^{o}C overnight. On the following day, the tube was spinned at maximum speed for 10 – 30 minutes in refrigerated centrifuge and the ethanol was removed. Four hundred microliter (400µl) of 70% cold ethanol was added and spinned at maximum speed for 5 minutes at 4^{o}C to precipitate the DNA because DNA is not miscible in alcohol. This step was repeated again to get more DNA precipitated and the salt totally removed. All traces of ethanol was removed by spinning the tube for 30 seconds at high speed and the DNA was dry out by leaving the tube open for 3 – 10 minutes. The pellet was re-suspended in 50µl sterile water for further analysis. Presence of DNA was confirmed by electrophoresing on an agarose gel containing ethidium bromide dye and checking under UV light.

Polymerase chain reaction

Amplification of resistant DNA fragments was performed using Applied Biosystems Taq DNA polymerase (Courtaboeuf, France) which is a Multiplex PCR kit. For reaction set-up, the PCR tube was placed on an ice pack and the following items were added to each isolate for single reaction: (a) Taq polymerase master mix (b) primers (forward and reverse) (c) template DNA and (d) distilled water. Five primers with known molecular weights were used to amplify DNAs from thirteen bacterial isolates to determine which bacteria gave PCR amplicons of the same size as the primer. Primers used in the multiplex PCR are listed in Table 1 below. The set-up was loaded on a PCR machine, PTC-100, Programmable Thermal Controller (M J. Research, Inc., USA). Thermocycling conditions for 35 cycles were as follows: pre-denaturation at 95^{o}C for 5 minutes; denaturation at 95^{o}C for 30 seconds; optimized annealing temperature for 30 seconds at 52^{o}C; extension for 1 minute at 72^{o}C followed by a final extension at 72^{o}C for 5 minutes. PCR products were resolved by gel electrophoresis. The process was repeated for positive isolates.

Table 1: PRIMERS FOR ANTIBIOTIC RESISTANCE GENES

S/N	Primer name	Sequence (5′→3′)	Target region	PCR product size (bp)	Annealing temp (°C)	References	Antibiotics resistance To
1	TEM-F TEM-R	ATTCTTGAAGACGAAAGGGC ACGCTCAGTGGAACGAAAAC	bla_{TEM}	1,150	60	Belaaouaj *et al.*, (1994)	Beta-lactams
2	*spvC*-F *spvC*-R	ACTCCTTGCACAACCAAATGCGGA TGTCTTCTGCATTTCGCCACCATCA	*spvC*	577	-	Ziemer and Steadham (2003)	-
3	SHV-F SHV-R	CACTCAAGGATGTATTGTG TTAGCGTTGCCAGTGCTCG	bla_{SHV}	885	52	Pitout *et al.*, (1998)	Cephalosporins
4	AacC3-F AacC3-R	CACAAGAACGTGGTCCGCTA AACAGGTAAGCATCCGCATC	*aac(3)-lll*	185	60	Van de Klundert and Vliegenthart, (1993)	Gentamicin
5	QnrSm-F QnrSm-R	GCAAGTTCATTGAACAGGGT TCTAAACCGTCGAGTTCGGCG	*qnrS*	428	54	Cattoir *et al.*, (2007)	Quinolone

Agarose gel electrophoresis

To confirm that DNA has actually been extracted, the DNA suspensions were subjected to agarose gel electrophoresis as follows: One percent (1%) agarose gel was prepared by dissolving 1g of agarose powder in 2ml of ten times concentration of Tris acetate ethylene diamine tetra acetate (TAE) buffer and 98ml distilled water in a 250ml beaker. This was heated in a microwave to dissolve the agarose. The gel was stained with 20µl of 5ml ethidium bromide dye and mixed. The gel was casted onto a gel electrophoresis tank with the combs inserted at the red bands to ensure easy view of the well and was allowed for 30 minutes to solidify. The casting combs were removed and 5µl of gel tracking dye (bromophenol blue) plus 15µl of the PCR products (making 20µl) were gently mixed and loaded into the well with the standard (Bioneer, 100 bp Plus DNA Ladder, Alameda, USA). The electrophoresis tank was covered and the electrodes connected to the power source for separation of sample based on molecular weight from cathode (-) to anode (+) at 100 mV for 45 minutes. At the completion of the electrophoresis, the gel was removed and viewed under a trans-illumination UV light (Gel Doc, Italy). The band pattern of the DNA fragments was then pictured and documented.

RESULTS

Results of molecular analysis of thirteen (13) resistant bacterial strains isolated from River Sokoto are presented in Fig. 1 as shown below:

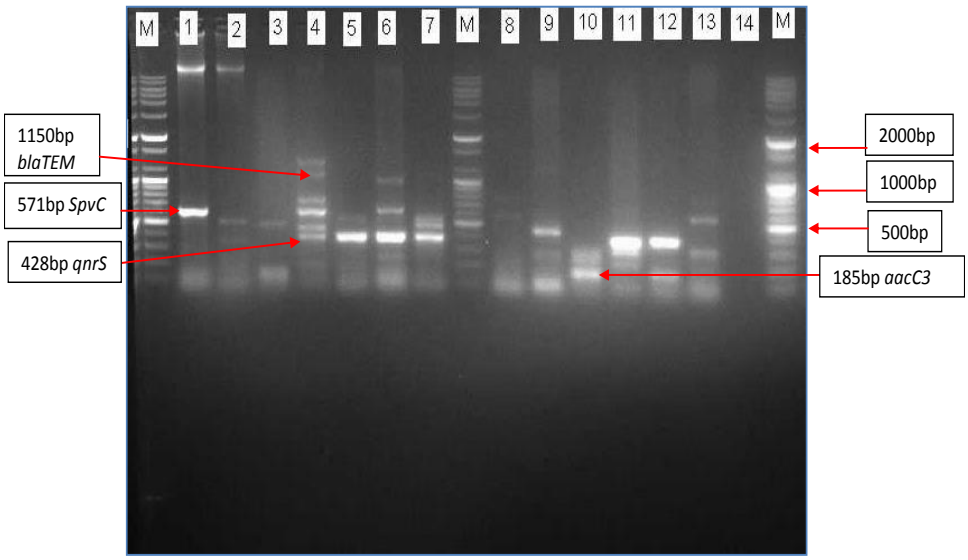

Figure 1: Agarose gel electrophoresis (1%) used for the separation of multiplex PCR products.
M = molecular weight marker (100 bp ladder; Bioneer, USA).

Table 2: Molecular Analysis of Resistant Bacteria Isolated from River Sokoto

Lane	Isolate	Number of Band	Band Size (bp)	Resistance Plasmids Detected
1	*Salmonella typhi*	2	571; > 10,200	*spvC*; Non-specific
2	*E. coli*	4	185; 300; 500; >10,200	*aacC3*;Non-specific
3	*Shigella flexneri*	2	185; 490	*aacC3*;Non-specific
4	*Pseudomonas aeruginosa*	6	428; 500; 571; 700; 1,150; 1,200	$bla_{TEM;}$ *spvC*; *qnrS*
5	*Staphylococcus aureus*	1	428	*qnrS*
6	*Streptococcus faecalis*	3	428; 500; 1000	*qnrS*; Non-specific
7	*Bacillus subtilis*	2	428; 500	*qnrS*
8	*Enterobacter cloacae*	1	185	*aacC3*
9	*Klebsiella oxytoca*	2	300; 500	Non-specific
10	*Staph saprophyticus*	2	185; 300	*aacC3*; Non-specific
11	*Providencia rettgeri*	2	400; 428	Non-specific; *qnrS*
12	*Klebsiella pneumonae*	2	185; 428	*aacC3*;*qnrS*
13	*Enterobacter aerogenes*	3	185; 300; 600	*aacC3*; Non-specific
14	-	0	-	Negative control

Table 2 shows the molecular analysis of resistant bacteria isolated from River Sokoto. Aminogglycoside resistance genes (*aacC3*) were detected in the majority of the isolates namely *E. coli* (lane 2), *Shigella flexneri* (lane 3), *Enterobacter cloacae* (lane 8), *Staphylococcus saprophytica* (lane 10) and *Enterobacter aerogenes* (lane 13). Virulence resistance genes (*spvC*) were detected in *Salmonella typhi* (lane 1) while quinolones resistance genes were detected in *Staphylococcus aureus* (lane 5), *Streptococcus faecalis* (lane 6), *Bacillus subtilis* (lane 7) and *Providencia rettgeri* (lane 11). Three different resistance genes which are β-lactam (bla_{TEM}), virulence (*spvC*) and quinolones (*qnrS*) were found in *Pseudomonas aeruginosa* (lane 4) while two resistance genes (*aacC3* and *qnrS*) were detected in *Klebsiella pneumoneae* (lane 12). However, non-specific resistance genes were detected in the majority of the isolates.

DISCUSSION

Plasmids harbor genes coding for specific functions including virulence factors and antibiotic resistance that permit bacteria to survive the hostile environment found in the host and resist treatment (Ramirez *et al.*, 2014). The uncontrolled use of antibiotics for empirical treatment of infectious diseases has been implicated as a cause of high prevalence of these antibiotics resistance (Olofsson, 2006). According to Odeyemi *et al.* (2015), another way by which these isolated bacteria can develop resistance to the various antibiotics is through the transfer of antibiotic resistant gene from one organism to another through their plasmids. This assertion was in agreement with the findings of this study as similar antibiotic resistance genes were detected in different plasmid carrying bacteria organisms which heightened the risk of bacterial infections in the study area as a result of wide spread of resistant strains to various commonly used antibiotics. In this study, Salmonella virulence plasmid genes (*spvC*) were detected in the *Salmonella typhi* isolates analysed. These virulence genes must have been the determinant genes for the

resistance earlier observed on *Salmonella typhi* isolates in the antibiotic sensitivity test. Salmonella plasmid virulence genes have been detected in various Salmonella species (Carlson *et al*., 1999; Ziemer and Steadham, 2003; Carattoli, 2003). The β-lactam (bla_{TEM}) and quinolone (*qnrS*) resistance genes detected in the test *Pseudomonas aeruginosa* isolates and; the aminoglycoside (*aacC3*) and quinolone (*qnrS*) resistance genes detected in the test *Klebsiella pneumoneae* isolates, is not new. In several geographical regions, including Argentina, the USA and Southeast Asia, plasmid mediated quinolone resistance (PMQR) determinants have been reported at high prevalence in both human and animal isolates and are often associated with the expression of extended-spectrum β-lactamases (ESBLs) (Park *et al*., 2006; Deepak *et al*., 2009; Zhang *et al.*, 2012; Andres *et al.*, 2013; Liu *et al.*, 2013; Shaheen *et al.*, 2013). Incidence of plasmid coding antibiotic resistance bacteria isolates, some of which posses plasmids with very high molecular weight (> 10,200 bp) in this study, is in agreement with the studies of Akinyemi *et al*. (2006) and Odeyemi *et al*. (2015) in Nigeria. The presence of quinolone resistance genes in *Staphylococcus aureus*, *Streptococcus faecalis*, *B. subtilis* and *Providencia rettgeri*; and aminoglycoside resistance genes in *Staph saprophyticus* may account for the relative high level of resistance of the isolates to the test antibiotics, earlier reported in this study. Non-specific resistance genes were detected in *Klebsiella oxytoca* and as additional resistance gene in other isolates. This is as a result of the fact that the primers used in this study are not all-encompassing and other resistance determinant genes, not captured in the study might have been responsible.

CONCLUSION

This study revealed that the antibiotic resistance genes in resistant bacteria pathogens isolated from River Sokoto were mainly virulence, aminoglycoside and quinolones resistance genes. This occurrence will heighten the risk of bacterial infections and promote failure to antibiotic treatments in the study area as a result of wide spread of resistant strains to various commonly used antibiotics.

People in the community should therefore be counseled on the usage and dosage of antibiotics.

Scientists are also challenged on the need for development of new antibiotics to combat the infections caused by these resistant bacteria.

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REFERENCES

1. Alzahrani, A.M. and Gherbawy, Y.A. (2011). Antibiotic resistance in *Escherichia coli* strains isolated from water springs in Al-Ahsa Region. *Afr. J. Microbiol. Res*., 5(2): 123-130.
2. Akinyemi, K. O., Oyefolu, A. O. B., Salu, O. B., Adewale, O. A. and Fasure, A. K. (2006). Bacterial pathogens associated with tap and well waters in Lagos, Nigeria. *East Cent. Afr. J. Surg.* **11(1):** 110-117.
3. Andres, P.; Lucero, C.; Soler-Bistue, A.; Guerriero, L.; Albornoz, E.; Tran, T.; Zorreguieta, A.; Galas, M.; Corso, A. and other authors (2013).Differential distribution of plasmid-mediated quinolone resistancegenes in clinical enterobacteria with unusual phenotypes of quinolonesusceptibility from Argentina.*Antimicrob Agents Chemother* **57:** 2467–2475.
4. Barza M, Travers K (2002). Excess infections due to antimicrobial resistance: the "Attributable Fraction", In: The need to Improve Antimicrobial Use in Agriculture: Ecological and Human Health Consequences, M Barza and SL Gorbach (Eds.,), *Clin. Infect. Dis*., 34: 126-130.
5. Baquero F, Martinez JL, Canton R (2008). Antibiotics and antibiotic resistance in water environments. *Curr. Opin. Biotechnol.*, 19: 260 - 265.
6. Belaaouaj, A., Lapoumeroulie, C, Canica, M. M., Vedel, G., Nevot, P., Krishnamoorthy, R. (1994). Nucleotide sequences of the genes coding for the TEM-like /5-lactamases IRT-1 and IRT-2 (formerly called TRI-1 and TRI-2). *FEMS Microbiology* Letters **120**: 75-80.
7. Carattoli, A. (2003) Plasmid-mediated antimicrobial resistance in Salmonella enteric. *Curr.Issues Mol. Biol*. **5:** 113-122.
8. Carlson, S.A., Bolton, L.F., Briggs, C.E., Hurd, H.S., Sharma, V.K., Fedorka-Cray, P.J. and Jones, B.D. (1999) Detection of multiresistant *Salmonella typhimuriium* DT 104 using multiplex and fluorogenic PCR. Molecular and Cellular Probes **13:** 213-222.
9. Cattoir, V., Poirel, L., Rotimi, V., Soussy, C. J. and Nordmann, P. (2007). Multiplex PCR for detection of plasmid-mediated quinolone resistance qnr genes in ESBL-producing enterobacterial isolates. *J Antimicrob Chemother* **60**: 394–397.
10. Deepak, R. N.; Koh, T. H. and Chan, K. S. (2009). Plasmid-mediated quinolone resistance determinants in urinary isolates of *Escherichia coli* and *Klebsiella pneumoniae* in a large Singapore hospital. *Ann Acad Med Singapore* **38:** 1070–1073.
11. Dubey, J.P. (2009) Toxoplasmosis of Animals and Humans, Second ed. CRC Press, Boca Raton, FL.
12. Hamner, S., Tripathi, A., Mishra, R.K. (2006) The role of water use patterns and sewage pollution in incidence of water-borne/enteric diseases along the Ganges River in Varanasi, India. *International Journal of Environmental Health Research*, 16 (2):113–132.
13. Liu, B. T., Liao, X. P., Yue, L., Chen, X. Y., Li, L., Yang, S. S., Sun, J., Zhang, S., Liao, S. D. and Liu, Y. H. (2013). Prevalence of β-lactamase and 16S rRNA methylase genes among clinical *Escherichia coli* isolates carrying plasmid-mediated quinolone resistance genes from animals. *Microb Drug Resist* **19:** 237–245.
14. Marti, E., Jofre, J. and Balcazar, J.L. (2013). Prevalence of Antibiotic Resistance Genes and Bacterial Community Composition in a River Influenced by a Wastewater Treatment Plant**.** PLoS ONE **8(10):** http://dx.doi.org/10.1371/journal.pone.0078906
15. Odeyemi, A.T., Awokunmi, E.E., Adebayo, A.A., Fasoranti, O.F. (2015) Plasmid profile of multi-drug resistance bacteria isolated from available water sources and leachate samples from dumpsite at Ebira communities in Ekiti North Senatorial District, Ekiti State, Nigeria. *European Journal of Advanced Research in Biological and Life Sciences*. 3(1): 31 – 45.
16. Olofsson, S. (2006). Relation between dug exposure and selection of antibiotic resistant bacteria. Dissertation presented at the Faculty of Medicine, Upsalla University for the degree of Doctor of Philosophy.
17. Oyedeji, O., Olutiola, P.O., Owolabi, K.D., Adeojo, K.A. (2011). Multi-resistant faecal indicator bacteria in stream and

well waters of Ile-Ife City, Southwestern Nigeria: Public health implications. *J. Public Health Epidemiol.* **3(8):** 371-381.

18. Park, C. H., Robicsek, A., Jacoby, G. A., Sahm, D. and Hooper, D. C. (2006) Prevalence in the United States of *aac(69)-Ib-cr* encoding a ciprofloxacin-modifying enzyme. *Antimicrob Agents Chemother* **50:** 3953–3955.
19. Pitout, J.D.D., Thomson, K.S., Hanson, N.D., Ehrhardt, A.F., Moland, E.S. and Sanders, C.C. (1998) β-lactamases responsible for resistance to expanded-spectrum cephalosporins in *Klebsiella pneumoniae*, *Escherichia coli*, and *Proteus mirabilis* isolates recovered in South Africa. *Antimicrobial Agents and Chemotherapy* **42**: 1350–1354.
20. Ramirez, M.S.; Traglia, G.M.; Lin, D.L.; Tran, T. and Tolmasky, M.E. (2014) Plasmid-mediated antibiotic resistance and virulence in Gram-negatives: the *Klebsiella pneumoniae* paradigm. *Microbiol Spectr*. **2(5):** 1–15.
21. Shaheen, B. W., Nayak, R., Foley, S. L. and Boothe, D. M. (2013) Chromosomal and plasmid-mediated fluoroquinolone resistancemechanisms among broad-spectrum-cephalosporin-resistant *Escherichia coli* isolates recovered from companion animals in the USA. *J Antimicrob Chemother* **68:** 1019–1024.
22. Van De Klundert, J. and Vliegenthart, J. (1993). PCR detection of genes coding for aminoglycoside-modifying enzymes. In: Persing *et al.* (editors) Diagnostic Molecular Microbiology: Principles and Applications. AMS Press, Washington, D.C., pp. 547-552.
23. Zhang, Y.; Yang, J.; Ye, L.; Luo, Y.; Wang, W.; Zhou, W.; Cui, Z. and Han, L. (2012). Characterization of clinical multidrug-resistant Escherichia coli and *Klebsiella pneumoniae* isolates, 2007-2009, China. *Microb Drug Resist* **18:** 465–470.
24. Ziemer, C. J. and Steadham, S. R. (2003) Evaluation of the specificity of Salmonella PCR primers using various intestinal bacterial species. *Letters in Applied Microbiology* **37**: 463-469.