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EXTENDED-SPECTRUM BETA-LACTAMASE PRODUCTION AMONG AMPICILLIN-RESISTANT *ESCHERICHIA COLI* STRAINS FROM CHICKEN IN ENUGU STATE, NIGERIA

Chah, K.F.[1]*; Oboegbulem, S.I.[2]

[1]Department of Veterinary Pathology and Microbiology; [2]Department of Veterinary Public Health and Preventive Medicine Faculty of Veterinary Medicine University of Nigeria, Nsukka Enugu State, Nigeria

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ABSTRACT

One hundred and seventy-two ampicillin-resistant *E. coli* strains isolated from commercial chickens in Enugu State, Nigeria, were screened for beta-lactamase production using the broth method with nitrocefin® as the chromogenic cephalosporin to detect enzyme production. Beta-lactamase producing strains were further examined for extended-spectrum beta-lactamase (ESBL) production using the Oxoid combination discs method. One hundred and seventy (98.8%) of the 172 ampicillin-resistant *E. coli* strains produced beta-lactamase enzyme. Sixteen (9.4%) beta-lactamase producers were phenotypically confirmed to produce ESBLs. Six of the ESBL producing strains were only detected with ceftazidime versus ceftazidime/clavulanate combination while ten of the ESBL producers were detected with cefotaxime versus cefotaxime/clavulanate combination. Chicken may serve as a reservoir of ESBL-producing *E. coli* strains which could be transferred to man and other animals.

Key word: Ampicillin-resistant, *Escherichia coli*, beta-lactamase, extended-spectrum

INTRODUCTION

Beta-lactam antibiotics account for approximately 50% of global antibiotic consumption and this heavy usage has exerted considerable selection for resistance (13). Production of beta-lactamases is the commonest cause of resistance to β-lactam antibacterial agents among Gram-negative bacteria (21). These enzymes undermined the utilization of ampicillin and first and second-generation cephalosporins in the chemotherapy of infections caused by Gram-negative bacteria. To overcome the problems posed by the β-lactamase enzymes, third- and fourth-generation (extended-spectrum) cephalosporins were developed. Unfortunately, members of the Family Enterobacteriaceae have developed resistance to these extended-spectrum cephalosporins via production of extended-spectrum beta-lactamases (ESBLs) (6,9). Apart from the ESBL enzymes, resistance to expanded-spectrum cephalosporins in enterobacteria has also been found to be mediated by AmpC β-lactamases (20). These enzymes are resistant to cephalosporin/clavulanate combination.

Human clinical strains of *Escherichia coli* producing ESBLs have been described from different parts of the world (5,8,16, 21). In Nigeria, these enzymes have been reported in *Enterobacter* species from human patients in Lagos (1).

Escherichia coli strains resistant to extended-spectrum cephalosporins have been isolated from calves (2) and dogs (23). This study was undertaken to assess ampicillin-resistant *E. coli* strains isolated from chickens for β-lactamase and extended-spectrum β-lactamase production.

MATERIALS AND METHODS

Bacterial strains

One hundred and seventy-two ampicillin-resistant *Escherichia coli* strains isolated from commercial chickens (broilers and layers) in Enugu State, Nigeria were used in the study.

*Corresponding Author. Mailing address: Department of Veterinary Pathology and Microbiology; Faculty of Veterinary Medicine University of Nigeria, Nsukka Enugu State, Nigeria. E-mail: kfchah@yahoo.com

Detection of beta-lactamase production

Test *E. coli* strains were screened for β-lactamase production using the broth method (14). Two colonies of each of the test bacteria were picked from an over night nutrient agar culture plate and inoculated into 1ml of sterile nutrient broth. Inoculated broths were appropriately labeled and incubated at 37ºC for 18 hours. After incubation, four drops of nitrocefin(R) solution (Calbiochem, Germany), prepared as directed by the manufacturer, were added to the broth culture and observed for colour change within 30minutes. Nitrocefin(R) is a chromogenic cephalosporin that changes colour from yellow to red on hydrolysis. Beta-lactamase production was inferred when the broth turned red within 30minutes of addition of reagent.

Detection of extended-spectrum beta-lactamase (ESBL) expression by *Escherichia coli* strains

This was done by initially screening for susceptibility to two extended-spectrum cephalosporins (ceftazidime and cefotaxime; Oxoid, England). Presumptive ESBL-producers were subjected to Phenotypic Confirmatory test for ESBL production.

Susceptibility test

Beta-lactamase producing *E. coli* strains were screened for susceptibility to ceftazidime (30 µg) and cefotaxime (30 µg), using the standard disc diffusion method in accordance with the National Committee for Clinical Laboratory Standards guidelines (18). Test bacteria were grown on Nutrient agar for 18 hours at 37ºC and colonies of the bacteria were suspended in sterile normal saline and the inoculum density adjusted to 0.5 McFarland turbidity standards. Surfaces of Mueller-Hinton agar were flooded with the standardized bacterial suspension and excess fluid drained into a discard pot containing Isol® disinfectant. With a disc dispenser (Oxoid, Basingstoke, England), ceftazidime (CAZ-30 µg) and cefotaxime (CTX-30 µg) were placed on the inoculated plates. The plates were incubated at 37ºC for 24 hours. After incubation the diameters of zones of inhibition around each antibacterial disc were measured with a metre rule. Each test was performed in triplicate and the mean inhibition zone diameter determined and recorded to the nearest whole millimetre. The strains were interpreted as susceptible or resistant according to the NCCLS criteria. According to these criteria, an *Escherichia coli* strain with a mean inhibition zone size equal to or less than 20mm was considered resistant to ceftazidime (30 µg) while each strain with mean zone diameter of less or equal to 22 mm was considered resistant to cefotaxime (30 µg). Also, in accordance with NCCLS guidelines a strain that produced a zone size ≤22 mm with ceftazidime or ≤27 mm with cefotaxime was considered a presumptive or possible extended-spectrum beta-lactamase (ESBL) producer. These possible ESBL-producers were subjected to Phenotypic Confirmatory test for ESBL expression.

Phenotypic confirmation of ESBL production

Presumptive ESBL-producing *E. coli* strains were tested by the Oxoid Combination Disc method (3,14) to confirm ESBL production. This test was done in accordance with standard recommendations (18). Two pairs of combination discs (ceftazidime-30 µg versus ceftazidime/clavulanate -30/10 µg and cefotaxime-30 µg versus cefotaxime/clavulanate - 30/10 µg) were used for the test. These discs were placed on the surface of Mueller-Hinton agar inoculated with the standardized inoculum of the test bacteria. After 24hours incubation at 37ºC the inhibition zones produced were measured. Each test was performed in duplicate and mean zone size recorded to the nearest whole millimetre. An *E. coli* strain was interpreted as ESBL-producers if there was a difference in zone size of ≥5 mm between the combination disc compared to that of the cephalosporin alone.

RESULTS

Beta-lactamase production

Using the nitrocefin(R) solution, beta-lactamase production was detected in 170 (98.8%) of 172 ampicillin-resistant *Escherichia coli* strains screened.

Extended-spectrum beta-lactamase production

The distribution of the mean inhibition zone diameters around the two cephalosporin discs (ceftazidime-30 µg and cefotaxime-30 µg) for the 170 β-lactamase producing *E. coli* strains is presented in Fig. 1. As shown in the figure, the zone sizes ranged from 18 mm to 40 mm for ceftazidime and 18 mm to 45 mm for cefotaxime. Using NCCLS (18) breakpoints for susceptibility, 16 (9.4%) of the test strains were resistant to ceftazidime while 14 (8.2%) were resistant to cefotaxime. Of the 170 beta-lactamase producing strains, 52 (30.6%) were presumptively identified as ESBL-producers with ceftazidime while 62 (36.5%) were detected as possible ESBL-producers with cefotaxime. However, when both discs were taken into consideration, 76 (44.7%) of the strains were predicted as ESBL producers. The proportion of the beta-lactamase producing *E. coli* strains resistant to ceftazidime alone, cefotaxime alone or both discs is presented on Table 1.

The distribution of inhibition zone differences of the possible ESBL-producers is shown in Fig. 2. Out of the 52 presumptive ESBL-producers detected by ceftazidime, 6 (11.5%) were phenotypically confirmed as ESBL with ceftazidime versus ceftazidime/clavulanate combination discs while 10 (16.1%) of the 62 possible ESBL producing *E. coli* strains detected with cefotaxime were confirmed as ESBL-producers with cefotaxime versus cefotaxime/clavulanate discs. When both pairs of Oxoid combination discs were taken into consideration, 16 (21.1%) of the 76 possible extended-spectrum beta-lactamase producing *E. coli* strains were confirmed to express ESBL phenotype. Thus,

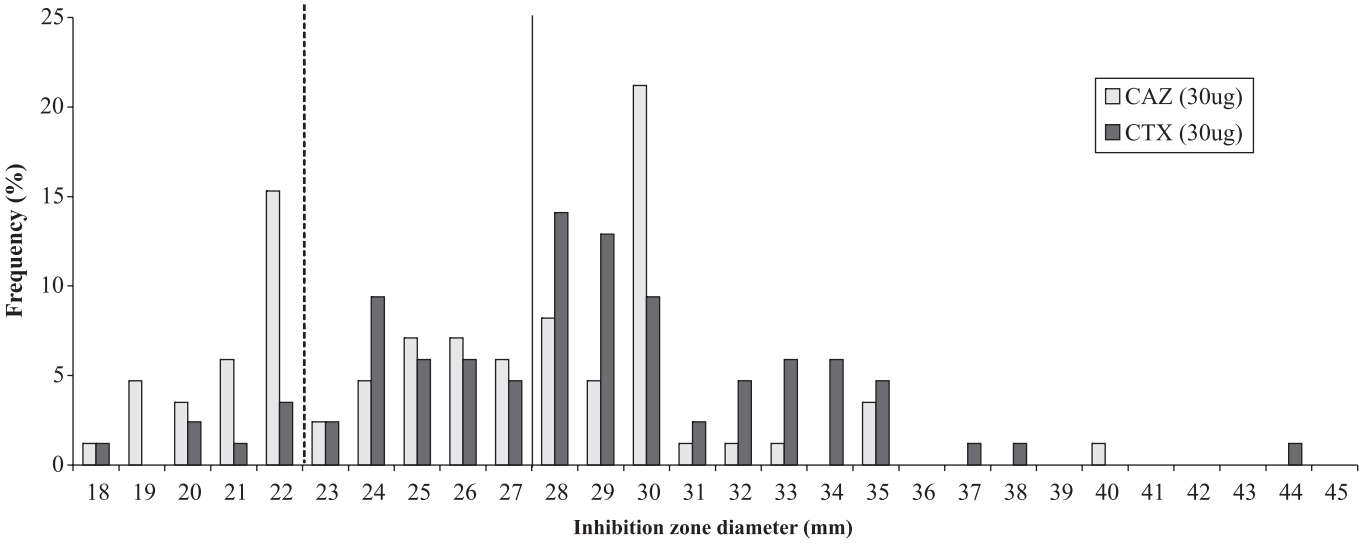

Figure 1. Inhibition zone diameters of ceftazidime (CAZ) and cefotaxime (CTX) against ampicillin-resistant *Escherichia coli* strains.

Table 1. Phenotypes of ampicillin-resistant *E. coli* strains isolated from chicken in Enugu State (N=170)

Phenotype	Phenotype detected with		
	Ceftazidime[1]	*Cefotaxime*[2]	*Both discs*[3]
Cephalosporin resistance[a]	10 (5.9)*	8 (4.7)	6 (3.5)
Presumptive ESBL-producer[b]	14 (8.2)	24 (14.1)	38 (22.4)
Confirmed ESBL-producer[c]	6 (3.5)	10 (5.9)	0 (0.0)
Cephalosporin/clavulanate resistance[d]	4 (2.4)	18 (10.6)	2 (1.2)

* = Figures in parenthesis represent percentage of beta-lactamase producing *E. coli* strains examined
a = Ceftazidime resistance (zone size ≤ 20 mm); cefotaxime resistance (zone size ≤ 22 mm)
b = Strain with zone size ≤ 22 mm with ceftazidime or zone size ≤ 27 mm with cefotaxime
c = Strain with cephalosporin/clavulanate vs plain cephalosporin zone difference ≥ 5 mm
d = Strains with cephalosporin/clavulanate vs plain cephalosporin zone difference of 0mm
1 = Phenotype detected with ceftazidime but not with cefotaxime
2 = Phenotype detected with cefotaxime but not with ceftazidime
3 = Phenotype detected with ceftazidime and also with cefotaxime

16 (9.4%) of the 170 beta-lactamase producing *E. coli* strains examined were ESBL producers. Out of the 76 possible ESBL producing strains, 24 (31.6%) were unaffected by at least one of the test cephalosporin/clavulanate discs as evidenced by zero zone difference between the cephalosporin versus cephalosporin/clavulanate combination discs.

The proportion of the beta-lactamase producing *E. coli* strains presumptively identified as ESBL-producer and phenotypically confirmed as ESBL-producers with either ceftazidime versus ceftazidime/clavulanate, cefotaxime versus cefotaxime/clavulanate or both combination discs is presented on Table 1. The inhibition zones produced around combination discs by ESBL-producing *E. coli* strains are shown in Figs. 3 and 4.

DISCUSSION

Antibacterial preparations containing β-lactams, particularly ampicillin, are widely used for prophylaxis and chemotherapy of avian bacterial infections in Nigeria (4). Heavy usage of β-lactam antibacterials exerts considerable selection for resistance to this class of antibacterial agents (15). In this study 98.8% of

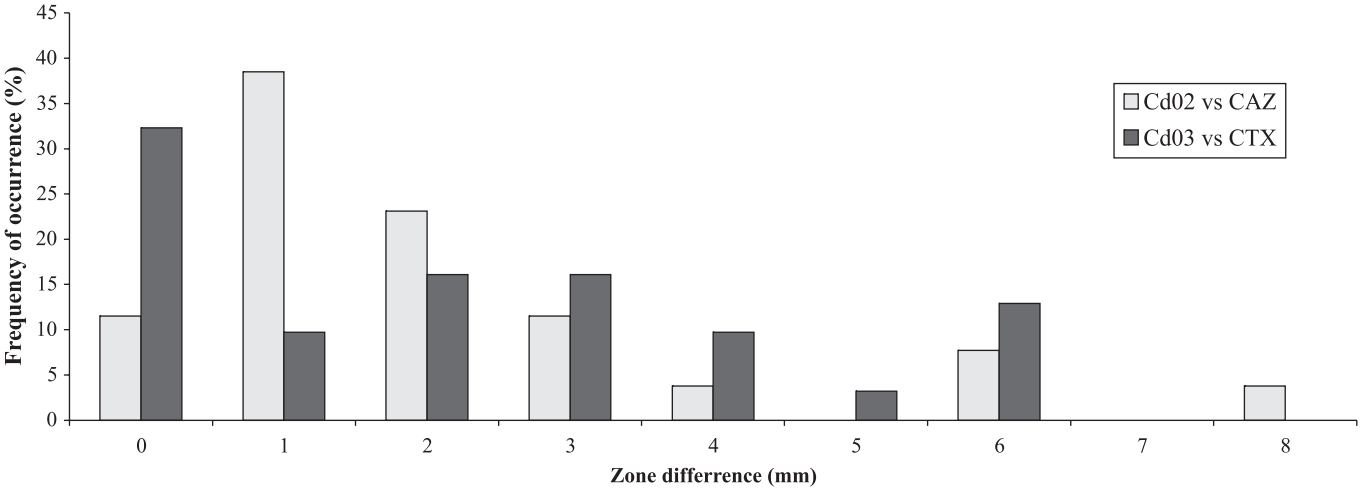

Figure 2. Inhibition zone differences produced by test cephalosporins alone and in combination with clavulanate.

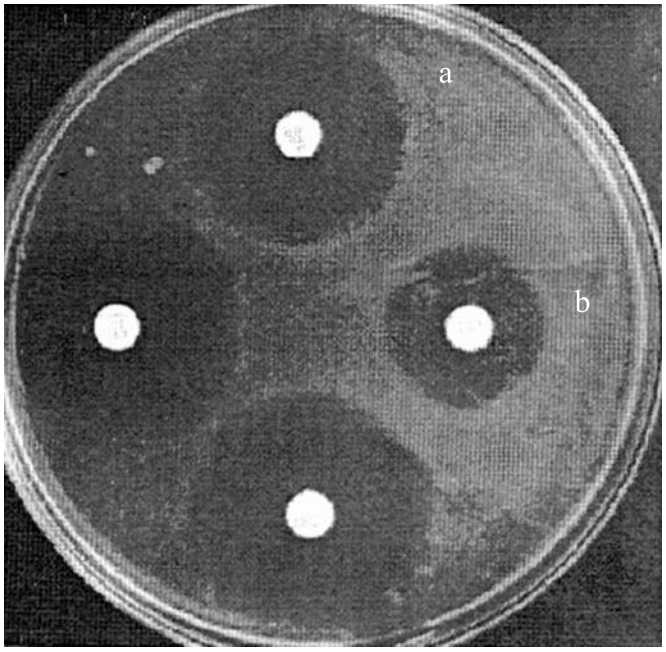

Figure 3. Extended-spectrum beta-lactamase producing *E. coli* strain.Confirmed with ceftazidime-clavulanate (a) versus ceftazidime alone (b).

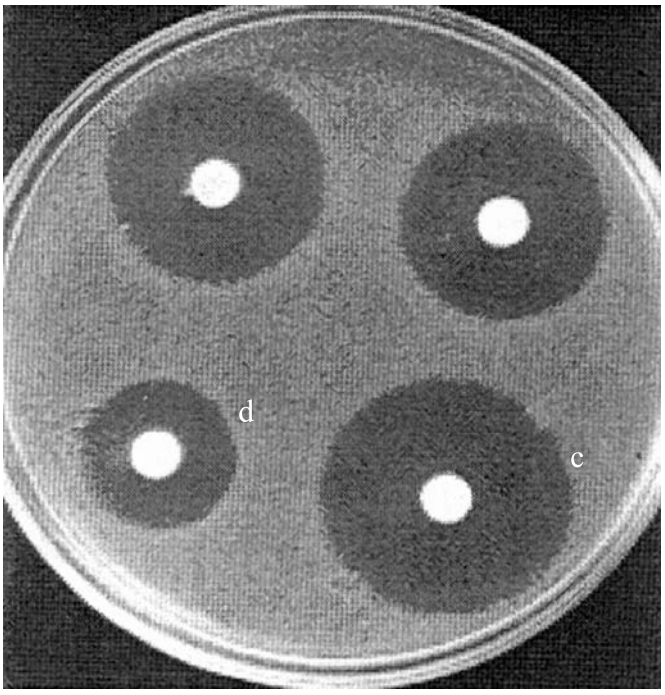

Figure 4. Extended-spectrum beta-lactamase producing *E. coli* strain. Confirmed with cefotaxime- clavulanate (c) versus cefotaxime alone (d).

the 172 ampicillin-resistant *E. coli* strains tested were beta-lactamase producers, while only 2 (1.2%) were non-producers. This observation supports previous reports (11,12) that resistance to β-lactams in *E. coli* is mainly due to production of beta-lactamases. These enzymes hydrolyze the cyclic amide bond in the β-lactam molecule leading to the formation of penicilloic acid or cephalosporoic acid (7). These products are unable to bind to penicillin-binding proteins (PBPs) located on the bacterial cytoplasmic membrane and therefore cannot inhibit

bacterial cell wall formation. However, it has been pointed out other mechanisms mediating resistance to β-lactams include porin deficiency, modification of PBPs, and permeability barrier and efflux pumps (19). Resistance to ampicillin among the 2 non-β-lactamase producing *E. coli* strains may be due to one of the above resistance mechanisms.

Although 52 (30.6%) and 62 (36.5%) of the β-lactamase producing *E. coli* strains were predicted as ESBL producers by ceftazidime and cefotaxime respectively, only 16 (9.4%) were phenotypically confirmed as ESBL producers. This is similar to the observations of previous workers (5,8). This underscores the need for phenotypic confirmation of ESBL production using Oxoid combination Disc method or other sensitive methods. In the present study, none of ESBL-producing strains confirmed with ceftazidime were identified as such with cefotaxime and vice versa. This observation supports previous reports that *E. coli* strains elaborate various types of ESBL enzymes with different substrate profiles (10).

In the present study a greater proportion of the ESBL-producers were detected with cefotaxime alone versus cefotaxime/clavulanate discs than with ceftazidime versus ceftazidime/clavulanate discs. This finding is contrary to the observations of other investigators (8,10,17). However, the finding in this study that when both pairs of the combination discs were taken into consideration, a higher proportion of ESBL-producing strains were detected than the rate recorded for either pair taken separately is similar to the report of M'Zali *et al*. (17) in which 86%, 65.5% and 93% of ESBL-producing members of the family Enterobacteriaceae were detected by discs containing ceftazidime, cefotaxime and both agents taken into consideration respectively. Optimal substrate profile varies from one ESBL-producer to another (22). This may explain the differences in the ESBL detection rates by the different cephalosporins. The overall 9.4% detection rate of ESBL production among the 170 lactamase producing *E. coli* strains examined may not represent the actual prevalence of ESBL among the *E. coli* isolates studied. This is because some of the ESBL producers might have escaped detection by the Oxoid combination discs used in this study. It is therefore possible that the true prevalence of extended-spectrum beta-lactamase producers among *E. coli* strains in the poultry population in the state may be higher than the value recorded in this study. Hence, inclusion of a wide variety of cephalosporins (such as cefpodoxime, aztreonam, ceftriaxone, cehpalothin etc) in the screening panel could possibly increase the rate of detection of ESBL-producing *E. coli* strains. Although the occurrence of a wide variety of ß-lactamases in South Africa was believed to reflect over use of the newer extended spectrum cephalosporins in medical practice in that country (21), in Nigeria, these agents are not used in veterinary practice. Thus, the presence of ESBL producing *E. coli* strains in poultry in the country may not be related to overuse of these agents. However, ampicillin is widely used in poultry production in the country (4) and this agent may provide a selective pressure favouring the emergence of *E. coli* strains that produce ESBL enzymes.

Results of this study have shown that ESBL producing *E. coli* strains are present in the chicken population in Nigeria. Chicken may therefore serve as reservoir of ESBL-producing *E. coli* strains, which could be transferred to humans and other animals.

RESUMO

Produção de beta-lactamase de espectro expandido por cepas de *Escherichia coli* resistentes a ampicilina isoladas de frango em Enugu State, Nigéria

Cento e setenta e duas cepas de *Escherichia coli* resistentes a ampicilina isoladas de frangos em Enugu State, Nigéria, foram avaliadas quanto à produção de beta-lactamase através do uso de método em caldo com nitrocefin® como indicador cromogênico da produção da enzima. Em seguida, as cepas produtoras de beta-lactamase foram examinadas quanto à produção de beta-lactamase de espectro expandido (ESBL) através do método de discos combinados Oxoid. Entre as cepas de *Escherichia coli* resistentes a ampicilina, cento e setenta (98,8%) produziram beta-lactamase. Testes fenotípicos indicaram que dezesseis (9,4%) das cepas produtoras de beta-lactamase produziram ESBL. Seis cepas produtoras de ESBL foram detectadas apenas com a combinação ceftazidima versus cefotaxime/clavulanato, enquanto dez cepas produtoras de ESBL foram detectadas com a combinação cefotaxime versus cefotaxime/clavulanato. Frangos podem ser reservatório de cepas de *E.coli* produtoras de ESBL, que podem ser transferidos para o homem e outros animais.

Palavras-chave: *Escherichia coli*, resistência a ampicilina, beta-lactamase, espectro expandido

REFERENCES

1. Aibinu, I.E.; Ohaegbulam, V.C.; Adenipekun, E.A.; Ogunsola, F.T.; Odugbemi, T.O.; Mee, B.J. (2003). Extended-spectrum β-lactamase enzymes in clinical isolates of Enterobacter species from Lagos, Nigeria. *J. Clin. Microbiol.*, 41: 2197-2206.
2. Bradford, P.A.; Petersen, P.J.; Fingerman, I.M.; White, D.G. (1999). Characterization of expanded-spectrum cephalosporin resistance in *E. coli* isolates associated with bovine calf diarrhoeal disease. *J. Antimicrob. Chemother.*, 44: 607-610.
3. Carter, M.W.; Oakton, K.J.; Warner, M.; Livermore, D.M. (2000). Detection of extended-spectrum β-lactamases in Klebsiellae with Oxoid Combination Disc Method. *J. Clin. Microbiol.*, 38: 4228-4232.
4. Chah, K.F.; Nweze, N.E. (2001): Antibiotic use in poultry production in Nsukka, Southeast Nigeria. *Proc. Nig. Soc. Anim. Prod.*, 26: 69-72.
5. Emery, C.L.; Weymouth, L.A. (1997). Detection and clinical significance of extended -spectrum β-lactamases in a Tertiary care Medical center. *J. Clin. Microbiol.*, 35: 2061-2067.

6. DuBois, S.K.; Marriott, M.S.; Amyes, S.G.B. (1995). TEM- and SHV-derived extended-spectrum β-lactamases: relationship between selection, structure and function. *J. Antimicrob. Chemother*., 35: 7-22.
7. Georgepa-padakou, N.H. (1993). Penicillin binding proteins and bacterial resistance to β-lactams. *Antimicrob. Agents Chemother.*, 37: 2045-2053
8. Jacoby, G.A.; Han, P. (1996). Detection of extended spectrum β-Lactamase in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli. J. Clin. Microbiol.*, 34: 908-911.
9. Jacoby, G.A. (1997). Antimicrobial resistance: extended-spectrum beta-lactamases and other enzymes providing resistance to oxyimino-beta-lactams. *Infect. Dis. Clin. North Am.*, 11: 875-887.
10. Katsanis, G.P.; Spargo, J.; Ferraro, M.J.; Sutton, L.; Jacoby, G.A. (1994). Detection of *Klebsiella pneumoniae* and *Escherichia coli* strains producing extended spectrum β-lactamase. *J. Clin. Microbiol.*, 32: 691-696.
11. Ling, T.K.; Lyon, D.J.; Cheng, A.F.B.; French, G.L. (1994). In vitro antimicrobial susceptibility and β-Lactamases of ampicillin-resistant *Escherichia coli* in Hong Kong. *J. Antimicrob. Chemother.*, 34: 65-71.
12. Liu, P.Y.F.; Gur, D.; Hall, L.M.C.; Livermore, D.L. (1992). Survey of the presence of β-Lactamases among 1000 Gram-Negative bacilli isolated consecutively at the Royal London Hospital. *J. Antimicrob. Chemother*., 30: 429-447.
13. Livermore, D.M. (1998). β-lactamase-mediated resistance and opportunities for its control. *J. Antimicrob. Chemother.*, 41: S25-S41.
14. Livermore, D.M.; Brown, D.F. (2001). Detection of β-lactamase-mediated resistance. *J. Antimicrob. Chemother.*, 48: 59-64.
15. Livermore, D.M. (2003). Bacterial resistance; Origins, epidemiology and impact. *Clin. Infect. Dis.*, 36: S11-23.
16. Mirza, S.H.; Salman, M.; Khurshid, U.; Wiqar, M.A. (2006). CTX-M ESBL enzymes in *Escherichia coli* from urology patients in Rawalpindi, Pakistan. *J. Pak. Med. Assoc.*, 56: 576-578.
17. M'Zali, F.H.; Chanawong, A.; Kerr, K.G.; Birkenhead, D.; Hawkey, P.M. (2000). Detection of extended-spectrum β-lactamases in members of the family Enterobacteriaceae: comparison of the MAST DD test, the double disc and the E-test. *J. Antimicrob. Chemother*., 45: 881-885.
18. NCCLS (2002). National Committee for Clinical Laboratory Standards. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Approved Standard - Second Edition. NCCLS document M31-A2.Wayne, Pennsylvania, USA.
19. Nikaido, H. (1996). Multidrug efflux pumps in Gram-negative bacteria. *J. Bacteriol*., 179: 5853-5859.
20. Papanicolaou, G.A.; Medeiros, A.A.; Jacoby, G.A. (1990). Novel plasmid mediated β-lactamase (MR-1) conferring resistance to oxyimino- and ∝-methoxy β-lactams in clinical isolates of *Klebsiella pneumoniae*. *Antimicrob. Agents Chemother.*, 34: 2200-2209.
21. Pitout, J.D.D.; Thomson, K.S.; Hanson, N.D.; Ehrhardt, A.F. (1998). β-Lactamase responsible for resistance to expanded spectrum cephalosporin in *Klebsiella pneumoniae, Escherichia coli, and Proteus mirabilis* isolates recovered from South Africa. *Antimicrob. Agents Chemother*., 42: 1350-1354.
22. Quinn, J.P.; Miyashiro, D.; Sahm, D.; Flamm, R.; Bush, K. (1989). Novel plasmid mediated β-lactamase (TEM-10) conferring selective resistance to ceftazidime and aztreonam in clinical isolates of *Klebsiella pneumoniae*. *Antimicrob. Agents Chemother.*, 33: 1243-1244
23. Warren, A.L.; Townsend, K.M.; King, T.; Moss, S.; O'Boyle, D.; Yates, R.M.; Trott, D.J. (2001). Multi-drug resistant *Escherichia coli* with extended-spectrum β-lactamase activity and fluoroquinolone resistance isolated from clinical infections in dogs. *Aust. Vet. J*., 79: 621-623.