

Comparison of Sensitivity and Specificity of Double Disc Synergy Test and Disc Replacement Method in Detecting Extended Spectrum Beta-Lactamase Producing *Escherichia coli* From Livestock and Herders

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ABSTRACT

Sensitivity and specificity are statistical measures used to evaluate the performance of diagnostic tests, including phenotypic screening tests. The emergence and dissemination of extended spectrum beta-lactamase (ESBL) producing bacteria of food-animals and humans origin are responsible for numerous infections with challenging treatment and control measures which calls for considerable concern of both Medical and Veterinary practitioners globally. The aim of this study was to detect phenotypically, the ESBL production by *E. coli* from Livestock and Herders with view to evaluating the sensitivity and specificity of two different ESBL phenotypic screening tests. Out of the 181 (15.7%) samples that yielded positive growths, 111 (61.3%) of them were *E. coli* isolates and of these, 91 (81.9%) were multidrug resistant out of which 37(40.7%) and 20 (22.0%) were phenotypically detected as ESBL producing phenotypes by DDST and DRM protocols respectively. This study revealed significant difference ($P < 0.05$) between the two phenotypic methods. Calves, lambs and terminally aged cattle / sheep as well as young herders / terminally aged herders in closed-proximity to their livestock and those living in over-crowded environment were mostly infected by the ESBL producing *E. coli*. Therefore, proper herd size and sanitary measures be adopted and maintained in farms where calves and Lambs less than 1 year are kept. Herders, below 10 years old and terminally old herders be properly monitored and those with diarrhoea be promptly treated. Herders with known history of prolonged hospitalization be examined for nosocomial infections by ESBL producing bacteria for better management.

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INTRODUCTION

Sensitivity and specificity are statistical measures used to evaluate the performance of diagnostic tests, including ESBL phenotypic screening tests. This refers to the ability of a particular protocol to correctly detects all the samples/ individuals that have the disease condition being tested for; which also refers to as the portion of the true-positives detected by the test out of all the positives while specificity refers to the ability of a particular protocol to correctly detects all the samples/ individuals that do not have the disease condition being tested for. This also refers the portion of the true-negatives detected by the test out of all the negatives (Arakawa *et al.*, 1997).

The emergence and dissemination of extended spectrum beta-lactamase (ESBL) producing bacteria of food-animals and humans origin are responsible for numerous infections with challenging treatment measures which calls for reliable ESBL phenotypic screening methods which will stand the taste of time for both Medical and Veterinary practitioners globally. Extended Spectrum Beta-Lactamases (ESBLs) are enzymes often located on the plasmids mostly of gram-negative bacteria and are capable of hydrolyzing penicillin, broad spectrum cephalosporins and monobactams (Alsterlund *et al.*, 2009).

Enterobacteraeae including *E. coli* have been responsible for numerous outbreaks of infections throughout the world with challenging treatment measures (Hawser *et al.*, 2009). *Escherichia coli* are Gram-negative, anaerobes, non-spore

forming, typically rod-shaped, about 2.0 micrometer (μm) long, 0.5 μm in diameter and cell volume of 0.6–0.7 μm^3 (Kubitschek, 1990). Although *E. coli* are usually harmless microbes but some strains are the most common cause of community-acquired bacteremia and also the fifth common cause of nosocomial infections (Friendman *et al.*, 2002). The pathogenic serotypes of *E. coli* are defined by their somatic antigen (O) and flagella antigen (H). *E. coli* have been identified to cause Coli-septicaemia, Coli-bacillosis, Coli-mastitis and Urinary tract infection (UTI) (Nicolas-Chanoine *et al.*, 2008).

In the North Eastern States of Nigeria, ESBL-Producing *E. coli* isolates have been reported to be on the increased resulting to rise in morbidity and mortality rates in livestock and herders (AL-Agamy *et al.*, 2006); (Peirano *et al.*, 2010). To check this rampantly growing trend, various phenotypic screening approaches having high sensitivity and specificity have been developed to detect ESBL production in clinical samples. Consequently, Several phenotypic ESBL detection methods including the commonly used Double Disc Synergy Test (DDST) and Disc Replacement Method (DRM) have sometimes been used simultaneously for detection of ESBL in clinical samples (Welch *et al.*, 2002).

However, there is paucity of information on comparison of their performance in terms of sensitivity and specificity in phenotypic screening of ESBL- producing *E. coli* isolates in north-Eastern States of Nigeria. Therefore, this study have been designed to re-assesse and compare the sensitivity and

specificity of DDST and DRM in detecting ESBL-producing *E. coli* isolates in livestock and herders in this sub-region of the country which would ultimately provide valuable knowledge to clinicians and researchers working towards effective antibiotics stewardship and infection control measures.

MATERIALS AND METHODS

Description of the Study Area

The study area is located in the North-East Sub-region of Nigeria, covering three (3) Livestock populated States of Adamawa, Bauchi and Taraba.

Adamawa State lies between latitude 7° and 11° North and between Longitude 110° and 140° East, covering a land mass of about 38,741 km², and currently having twenty-one (21) Local Government Areas namely: Demsa, Fufure, Ganye, Girei, Gombi, Guyuk, Hong, Jada, Lamurde, Mayo- Belwa, Madagali, Maiha, Michika, Mubi North, Mubi, South, Numan, Shelleng, Song, Toungo, Yola-North and Yola-South with Yola as its State Capital (Adebayo, 1999). Out of which twelve (12) Local Government Areas (Dems, Fufure, Girei, Gombi, Guyuk, Lamurde, Mayo-Belwa, Numan, Shelleng, Song, Yola-North and Yola-South) were sampled while remaining nine (9) were not sampled because of reasons ranging from insecurity, difficult terrain and economic constraint. The State has a tropical wet and dry seasons, with dry season last for about five (5) months (November to March) while the wet season lasting for about seven (7) months (April to October) recording mean annual rainfall range of 700 mm to 1600 mm.

The State shares boundary with Taraba State in the south-west, Gombe State in its North-west and Borno state to the North. The State has an international boundary with the Republic of Cameroon along its Eastern part. Ecologically, the state is divided into guinea and Sudan savannah zones. Adamawa state has an estimated human population of 5,236,948 million people and about 80% of people of the state are engaged in large scale crops, and Livestock farming (Adebayo, 1998). The state is bordered is by seven (7) states; namely Kano and Jigawa to the north, Taraba and Plateau to the south, Gombe and Yobe to the east and Kaduna to the west. The state is known for its vast fertile grass land for Livestock rearing and crop production (Come to Nigeria Staff, 2011).

Bauchi State lies between latitude $9^{\circ}3'$ and $12^{\circ}3'$ North of the Equator with a total Land mass of 49,110 km² and presently having twenty (20) Local Government Areas with Bauchi city as its State Capital. These include: Alkaleri, Bauchi, Bogoro, Dambam, Darazo, Dass, Gamawa, Ganjuwa, Giade, Itas/ Gadau, Jama'are, Kaltungo, Kirfi, Misau, Ningi, Shira, Tafawa-Balewa, Toro, Warji and Zaki Local Governments respectively.

Out of these, 11 Local Government Areas (Alkaleri, Bauchi, Darazo, Dass, Gamawa, Ganjuwa, Kirfi, Misau, Ningi, Tafawa-Balewa and Toro) were sampled.

Geographically, the State has two distinct vegetation zones, namely Sudan Savannah which covers the southern part of the State where grasses with less uniformly bid tall trees are found and the Sahel Savannah (semi desert zone) starting from the middle of the State to its extreme North, where predominantly short grasses with isolated stands of thorny shrubs are found. The state is known for its vast fertile grass land for cattle rearing and crop production (Come to Nigeria Staff, 2011). The estimated human population for Bauchi state as at the year, 2020 was 7,540,663 million people. The people of state are predominantly involved in livestock production. Bauchi

State has a projected livestock population of 3,612,463 million comprising of Cattle, sheep, goats, rabbit and pigs.

The state is bordered is by seven (7) states; namely Kano and Jigawa to the north, Taraba and Plateau to the south, Gombe and Yobe to the east and Kaduna to the west.

Taraba state lies between Latitude $6^{\circ}30'$ and $8^{\circ}30'$ north of the Equator and between Longitude $9^{\circ}00'$ and $12^{\circ}00'$ east of the Greenwich Meridian with land mass of 60,291km². The State is bordered to the west by Nasarawa and Benue States, to the North-west by Plateau State, to the North by Bauchi and Gombe States, to the North-east by Adamawa State, and to the East and South by Republic of Cameroon. Taraba State lies largely within the middle of Nigeria and consists of undulating landscape dotted with a few mountainous features. These include the scenic and prominent Mambilla Plateau with an altitude of 1,800 meters (6000 ft) above sea level and has temperate climate all year round. The state lies largely within the tropic and has a vegetation of low forest in the southern part and grassland in the northern part. The major occupation of people of the State are Crop production and Livestock (Cattle, Sheep & Goat) rearing. Similarly, some farmers rear other Livestock like poultry, rabbit and pig farming in fairly large scale (Zeitlyn and Connell, 2003). According to Magnitude and trends, Taraba State projected Livestock population for 2018 was 23, 549,583 Million, comprising of 5,577,980 cattle, 3,061,666 sheep, 3,686,973M goats, 3,212,979M pigs and 8,009, 986M poultry respectively (Ayi et al., 2020).

Determination of Samples Size

The sample size was calculated by using the formula: $n = t^2 \times p(1-p) \div m^2$ from each sample source as described by FAO (1990). Where n = number of samples to be collected for this study, t = confidence level at 95% (standard value is 1.96), m = margin of error at 5% (standard value is 0.05). p = existing prevalence rate within the study area.

Since the prevalence rate within the study area is unknown, it was assumed to be 50% (0.5). Therefore sample size ' N ' = $1.96^2 \times 0.5(1-0.5) \div 0.05^2$

$n = 3.8416 \times 0.5 \times 0.5 \div 0.0025 = 384.16$. This figure was multiplied by 3, for the 3 states

Therefore, a total of 1152 samples comprising of 384 samples from each State within the study area were collected and processed for this study.

Study Population and Sampling Procedures

A cross-sectional descriptive studies were conducted on ESBLs producing *E. coli* from white Fulani breed of cattle, salami breed of sheep which is the most predominant breed in north-eastern Nigeria and their herders themselves in Adamawa, Bauchi and Taraba States located in the North-east Sub-Region of Nigeria were sampled. The sampling involved 384 of white Fulani breed of cattle (from 0 - 11 years of age; 384 of balami breed of sheep (0 - 9 years old and 384 of the herders (0 - 70 years of age (Pehlivanoglu et al., 2016) were sampled using the random sampling method. Swab samples were directly and non-repeatedly collected from the rectum of each sampling animals as follows:

The sampling animal was properly restrained and the tail was raised with one hand and a sterile swab stick was inserted in to the rectum directly in a rotatory manner until its touches the faeces in the rectum. The swab soiled-stick was removed and labeled. All remaining samples were collected using the same procedure. While stool samples from the herders were collected in to sterile sample bottles at the point of defecation by themselves. The bottles were properly labeled. The remaining samples from the herders were collected using the

same procedure. All samples were transported to ATBU postgraduate Microbiology Laboratory on ice-pack for culturing procedures.

Preparation of Culture Media, Enrichment, Inoculation and Incubation Procedures

The culture media [EMB and, MH agars (OXOID U.K)], employed for this study were prepared aseptically in ATBU Postgraduate Microbiology Laboratory according to the manufacturer's instructions. A 10% suspension of stool sample in buffered peptone water (Lab. M. UK) was prepared using a vortex vibrator and incubated at 37°C for 24 hours in aerobic condition. The swab was soaked in 10% buffered peptone water (PW) (Lab. M. UK) and incubated at 37°C for 24 hours. A loop full of the suspension of enriched swab samples was picked and inoculated on to Eosin Methylene Blue Agar (EMB agar OXOID, U.K) by streak method and incubated at 37 °C for another 24 hours aerobically for observation of growth.

Macroscopic and Microscopic Examinations

After 24 hours, inoculated cultures were grossly observed for bacterial growth and characteristic colonial appearance. Mixed cultures were sub-cultured to obtain pure cultures. The presumptive *E. coli* isolates were inoculated onto Mueller-Hinton agar slants and incubated at 37°C for 24 hours until growth was observed. These slants cultures were preserved in refrigerator for subsequent studies.

Slides prepared from pure colonies were first gram-stained and examined microscopically to determine the gram reaction of the isolates according to method developed by Hans Christian Gram (1884) and described by Naveena and Joy (2014).

Biochemical Identification Tests

The Pure culture isolates of *E. coli* were subjected to the four biochemical tests such as Indole (I), Methyl-Red (MR), Voges-Proskauer (Vp), and Citrate utilization represented by the Acronym (IMViC) which differentiate *E. coli* from other *Enterobacteria*. Other tests like Triple Sugar Iron (TSI) and Motility tests were also conducted as described by Naveena and Joy (2014). The characteristic isolates were identified on the basis of sugar utilization, IMViC reaction patterns and production of gas, ability to ferment lactose/sucrose in triple-sugar Iron (TSI) slants for Medium Sulphide, Indole and Motility (SIM), Methylene Red, Voges-Proskauer reaction and Citrates utilization (OXOID, UK).

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility tests were performed according to guidelines recommended by the Clinical Laboratory Standard Institute (CLSI, 2020) on Mueller Hinton agar plates (Oxoid). All discs for diffusion techniques were obtained from Oxoid with the following concentrations: Amikacin (25µg), Streptomycin (30µg), Amoxicillin (30µg), Cefotaxime (25µg), Cefotaxime (25µg), Ciprofloxacin (15µg), Ofloxacin (10 µg), Pefloxacin (25 µg) Bactrin (30ug) and Co-trimoxazole (30 µg).

0.5 McFarland standard suspension (inoculum) of the test isolate was made in normal saline using the McFarland vortex device and sterile swab sticks. The suspension of the inoculum was streaked uniformly as lawn on a Mueller Hinton Agar (MHA) plate. The required disks were placed at the appropriate place and incubated at 37°C for 24 hours.

Identification and Preservation of MDR *E. coli* Isolates

All *E. coli* isolates found resistant to three (3) or more classes of antibiotics were sorted out as multi-drug resistant (MDR) isolates and preserved in the refrigerator for subsequent studies.

Phenotypic Detection of ESBL Producing *E. coli* from MDR Isolates

The ESBL producing *E. coli* isolates were phenotypically detected using the Double Disc Synergy Test (DDST) and Disc Replacement Method (DRM) with view to comparing the sensitivity and specificity of the two protocols of phenotypic screening.

Double Disc Synergy Test (DDST)

The Double Disc Synergy Test was conducted as described by Jarlier *et al.*, (1988). *E. coli* NCTC 10418 was used as ESBL-negative control while *K. pneumoniae* ATCC 700603 was used as ESBL-positive control. The test isolates were evenly spread onto Mueller Hinton Agar plates and two antimicrobial discs were placed 30mm apart (center to center) with one of these containing amoxicillin/clavulanic acid and the other containing expanded spectrum cephalosporin (ceftriaxone, cefotaxime or ceftazidime) and incubated for 24 hours. Enhancement of zone of inhibition in between the discs indicates positive result. The presence of ESBL was confirmed by demonstrating synergy between cephalosporin and clavulanic acid with reduced halo.

Disc Replacement Method (DRM)

Here, the suspected ESBL producing *E. coli* isolates were subjected to phenotypic screening test using the Disc Replacement Method as described by Casal and Springler, (1990) Two (2) combined discs of amoxicillin/ clavulanic acid (Augmentin) (AMC 30ug, Oxoid England) were placed on prepared Muller-Hinton agar evenly inoculated with the testing isolates. After an hour at room temperature, the discs were removed and replaced at the spot of each, by Cefotaxime (CTX 30ug) and Ceftazidime (CAZ 30ug), inoculated at 37°C. After 24 hours the plates were read for evidence of ESBL production. Increased in diameter of zone of inhibition of > 5mm from the initial, confirmed ESBL production.

Determination of Sensitivity and Specificity of DDST and DRM Screening Tests

The sensitivity and specificity of DDST and DRM phenotypic screening tests were determined as developed by Jarlier *et al.*, (1988) and described by Arakawa *et al.*, (1997) as follows:

Sensitivity of any given test, is its ability to detect all the true-positive cases while specificity is its ability to detect all the true - negative correctly. Therefore, sensitivity is equal to True-Positive (TP) divided by True-Positive plus False-Negative (FN) multiplied by 100. The sensitivity and specificity of two category tests in medical settings (screening and definitive /diagnostic tests) can be determined as described by using the formula

$$\text{Sensitivity} = \frac{\text{TP}}{\text{TP} + \text{FN}} \times 100 \quad (1)$$

Whereas specificity is equal to True-Negative (TN) divided by True-Negative (TN) Plus false-positive (FP) multiplied by 100. $\text{Specificity} = \frac{\text{TN}}{\text{TN} + \text{FP}} \times 100 \quad (2)$

Where 'TP' is the true positive result of the gold standard test, 'TN' is true negative result of the gold standard test, 'FP' is false positive or the overlap between the screening test-positive and the gold standard test- positive results, 'FN' is the false negative or the over lap between the screening test-negative and the gold standard test-negative results.

Statistical Analysis

Chi-square test of relation (Fisher's test) was used to compare and determine the level of significance between the variables. By using SPSS computer software version 20.0

RESULTS AND DISCUSSION

Characteristics Bacteria isolated on EMB Agar from Livestock and Herders in Adamawa, Bauchi and Taraba States

Out of the 1152 samples collected, processed and inoculated on Eosin Methylene Blue (EMB) agar, 181 (15.7%)

comprising of 65 (5.6%), from Adamawa, 60 (5.2%), Bauchi and 56 (4.9%), Taraba had positive bacterial growths. Of the 181 positive cultures, 111 comprising of 39 (21.5%) from Adamawa, 38 (21.0%) from Bauchi and 34 (18.8%) from Taraba States, were *E. coli*, 19 comprising of 6 (3.3%) from Adamawa, 7 (3.9%) from Bauchi and 6 (3.3%) from Taraba States, were *Salmonella* species while 51 comprising of 20 (11.0%) from Adamawa, 15 (8.3%) from Bauchi and 16 (8.8%) from Taraba states, were *Shigella* species (Table 1).

Table 1: Distribution of Bacterial isolates from livestock and Herders in Relation to Sampling States

Sampling States	No. of Sample collected (N=1152)	No. (%) of positive cultures (n=181)	No. (%) of bacterial isolated (n=181)		
			<i>E. coli</i> (n=111)	<i>Salmonella</i> (n=19)	<i>Shigella</i> (n=51)
Adamawa	384	65(5.6)	39 (21.5)	6 (3.3)	20 (11.0)
Bauchi	384	60(5.2)	38 (21.0)	7 (3.9)	15 (8.3)
Taraba	384	56(4.9)	34 (18.8)	6 (3.3)	16 (8.8)

Although *E. coli* are usually harmless microbes but some strains are the most common cause of community-acquired bacteremia and also the fifth common cause of nosocomial infections (Friedman *et al.*, 2002). However, the pathogenic serotypes of *E. coli* are defined by their somatic antigen (O) and flagella antigen (H). *E. coli* have been identified to cause Coli-septicaemia, Coli-bacillosis, Coli-mastitis and Urinary tract infection (UTI) (Nicolas-Chanoine *et al.*, 2008 ; (Peirano *et al.*, 2010).

In this study, an overall prevalence rate of *E. coli* isolates in bot cattle, sheep and herders was 61.3% with relative prevalence of 21.5%, 21.0% and 18.8%, from Adamawa, Bauchi and Taraba States respectively. Macroscopically, 111(61.3%) plates with characteristic green metallic sheen colonies were presumptively identified as *E. coli*, while plates with transparent / colourless/amber colonies were presumptively identified as *Shigella* and plates having colourless colonies with black centre were presumptively identified as *Salmonella* species. Microscopically, all the 181 positive plates were gram-negative rods although some rods were shorter than others. The presumptive 111 *E. coli* colonies appeared to be long-rods while 70 isolates comprising of 19 *Salmonella* and 51 *Shigella* species were short-rods. The distribution analysis of *E. coli* isolates occurrence among the sampling States showed no significant variation suggesting that location may not be an important risk factor and *E. coli* organisms are ubiquitous in occurrence and can be disseminated to different geographical areas of the world like Europe, Asia, America, Korea, Middle east and Africa including Nigeria (Al-Agamy *et al.*, 2006).

Several previous studies revealed varying prevalence rates of *E. coli* across the world. Maikai and Akubo, (2018) reported a prevalence of 21.3% of *E. coli* in fresh fruits and vegetables at

Samaru, Kaduna State, Nigeria. Adzitey *et al.*, (2018) reported an overall prevalence of 20.18% *E. coli* from milk, milk

products and milkers hands at Tamale Metropolis, Ghana. All these prevalence are lower than the 61.3% recorded in this study suggest that the prevalence of *E. coli* is on the increase. However, these variations could be as result of sample size, type, source and sampling time as in this study swab samples were collected directly from the rectum of livestock/herders and *E. coli* is believed to be widespread gastro-intestinal tract commensals of man and animals resulting from food animal slaughter (Eckburg *et al.*, 2005).

Antimicrobial Resistance (AMR) Profile of *E. coli* Isolates from Livestock and Herders in this Study

Out of the 111 *E. coli* isolates subjected to antimicrobial susceptibility testings in relation to sample sources (Cattle, Sheep and Herders), 91 isolates comprising of 51 isolates of cattle, 23 of sheep and 17 of herders origin showed multiple drug resistance (MDR) to all classes of antibiotic used in this study. Among the beta-lactam class, (amoxicillin, ceftazidime and cefotaxime), amoxicillin recorded the highest MDR rate of 100% with herders isolates followed by 94.1% of cattle and 86.9% of sheep. Among the Flouroquinolones, ofloxacin recorded the highest MDR rate of 82.4% from the isolates of cattle, 65.2% from sheep and the lowest rate of 52.9% from herders respectively. From the Aminoglycosides class, Amikacin recorded the lowest MDR rate of 30.4% in sheep, followed by 35.3% from herders and the highest rate of 50.9% from the isolates of cattle respectively. Sulphonamides (Co-Trimoxazole and Trimethoprim/sulfamethoxazole), Co-Trimoxazole accounted for the highest MDR rate of 82.4% , 60.9% and 52.9% from cattle, sheep and herders respectively. Being in the same class, Trimethoprim/ sulfamethoxazole recorded similar MDR rate of 65.2%, 64.7% and 62.7% from the *E. coli* isolates of sheep, herders and cattle respectively (Table 2).

Table 2: Distribution of Antimicrobial Resistance Profile of *E. coli* Isolates from Livestock and Herders in this Study

Antibiotics (Concentrations)	No. of <i>E. coli</i> isolates tested (n=111)			No. (%) of MDR- <i>E. coli</i> isolates (n=91)		
	Cattle (n=64)	Sheep (n=27)	Herders(n=20)	Cattle (n=51)	Sheep (n=23)	Herders (n=17)
Amoxicillin (30µg)	64	27	20	48 (94.1)	20 (86.9)	17 (100)
Ceftazidime (25 µg)	64	27	20	40 (78.4)	16 (69.6)	10(58.8)
Cefotaxime (25 µg)	64	27	20	39 (76.5)	17 (73.9)	10 (58.8)
Ofloxacin (10 µg)	64	27	20	42 (82.4)	15 (65.2)	09 (52.9)

Antibiotics (Concentrations)	No. of <i>E. coli</i> isolates tested (n=111)			No. (%) of MDR- <i>E. coli</i> isolates (n=91)		
	Cattle (n=64)	Sheep (n=27)	Herders(n=20)	Cattle (n=51)	Sheep (n=23)	Herders (n=17)
Pefloxacin (25 µg)	64	27	20	41 (80.4)	14 (60.9)	09 (52.9)
Ciprofloxacin (15 µg)	64	27	20	36 (70.5)	16 (69.6)	13 (76.5)
Streptomycin (30 µg)	64	27	20	26 (50.9)	08 (34.8)	08 (47.1)
Amikacin (25 µg)	64	27	20	26 (50.9)	07 (30.4)	06 (35.3)
Sulphonamide (30 µg)	64	27	20	42 (82.4)	14 (60.9)	09 (52.9)
Trimethoprim(30µg)	64	27	20	32 (62.7)	15 (65.2)	11 (64.7)

Key : CLSI 2020

In this study, following the guidelines recommended by Clinical Laboratory Standard Institute (CLSI, 2020), 111 *E. coli* isolates were subjected to antimicrobial multidrug resistance testing at 37 °C, for 24 hours using four commonly prescribed classes of antibiotics (Aminoglycosides, Flouroquinolones, Sulphonamides and Beta-lactams). However, difference rates of susceptibility reactions were obtained. Multidrug resistant (MDR) refers to those *E. coli* isolates that showed resistance to at least 3 or more classes of antibiotics (Magiorakos *et al.*, 2012). The results were interpreted into three (3) categories as resistant, intermediate-susceptible (IS) and susceptible (S) respectively. Resistant strains of *E. coli* isolates were those which were able to grow in the presence and even on the antibiotic disc, while susceptible, were those inhibited by the drug at 37 °C within 24hrs of incubation with significant zones of inhibition. The results of the current study is consistent with the report of Mohammed *et al.* (2024). However, in between the two, was the intermediate-susceptible category (IS) which can be susceptible either by increasing the concentration of the drug, or incubation period or both.

The result of the antimicrobial susceptibility testing on *E. coli* isolates reveal concerned level of resistance to multiple classes of antibiotics. The highest resistance rates observed were among the Beta- Lactams (AMX, CAZ, CTX), moderately higher resistance rates were observed among the Flouroquinolones (OFL, PEF, CPX) and the Sulphonamides (TMP, SXT) suggesting a potential challenge in the treatment of infections due to gram negative bacterial. According to study conducted by Hooper and Jacoby, (2015), the increasing prevalence of antibiotics resistance in gram- negative bacteria, including *E. coli*, is a global concern that may amount to treatment failure and increased morbidity and mortality. These resistance mechanisms can be attributed to over-use/ mis-use of antibiotics as well as horizontal transfer of resistance genes among bacterial population. Despite the

high resistance rates observed in other antibiotic classes, aminoglycosides (amikacin & streptomycin), presented the lowest resistance rates of 33.3% and 37.7% respectively, which suggests that antibiotics of this class could be a drug of choice for treatment of infections by gram-negative bacteria. The low antibiotics resistance recorded by amikacin and streptomycin in this study is quite a promising finding and can be attributed to their ability to bind to the bacteria ribosome thereby disrupting the protein synthesis. This mechanism may make it more difficult for the organism to develop resistance as compared to the other classes antibiotics, or it could be indicative of limited pressure from the use of these antibiotics in clinical settings. By and large, the critical importance of prudent antibiotics use and infection control to combat the growing trend of antibiotic resistance in gram-negative bacteria pathogens is very crucial.

However, the average MDR rate (35.5%) recorded in aminoglycosides in this study is lower than the MDR rate of 48.4% reported by Phelivanoglu *et al.*, (2016). This suggests that antibiotics of the aminoglycosides class may likely be drug of choice for management and treatment of coliform infections in livestock and herders. This finding corroborates with the reports of Daga *et al.*, (2019).

Multidrug Resistance (MDR) Profile of *E. coli* Isolates from Livestock and Herders in this Study

Following subjection of 102 *E. coli* isolates comprising of 60 isolates of cattle, 23 isolates of sheep and 19 isolates of herders origin that showed antimicrobial resistance, a total of 91 (89.2%) isolates comprising of 51 of cattle, 23 isolates of sheep and 17 isolates of herders origin were tested to be MDR. Out of the MDR isolates, 38 were resistant to four classes of antibiotics and 53 isolates were resistant to three classes of antibiotics respectively. While 20 isolates were either resistant to one or two antibiotics class and as per definition of MDR, were considered as Non-MDR isolates (Table 3).

Table 3: Distribution of Multi-drug Resistance (MDR) Profile of *E. coli* Isolates from Livestock and Herders in this Study

Sample source	No. of <i>E. coli</i> isolates tested	No. (%) of MDR <i>E. coli</i> isolates resistant to 3&4 classes of antibiotics (n=91)		No. (%) of Non-MDR <i>E. coli</i> isolates; 2&1classes of antibiotics (n=20)	
		Four classes	Three classes	Two classes	One class
Cattle	64	27 (29.7)	24 (26.4)	09 (45.0)	04(20.0)
Sheep	27	05 (5.5)	18 (19.8)	00 (0.0)	03(15.0)
Herders	20	06 (6.6)	11 (12.1)	02 (10.0)	02 (10.0)
Total	111	38 (41.8)	53 (58.2)	11 (55)	09 (45.0)

Indeed, the discovery and development of antibiotics was by no doubt one of the greatest breakthroughs in the field of modern medicine. However, the emergence of bacterial resistance to antibiotics is frightening to the efficacy of many antimicrobial agents. Prescription of these antimicrobial agents are very common by both Medical and Veterinary

doctors and their used is questionable (Igbal *et al.*, 2017). Flouroquinolones, aminoglycosides, beta-lactams and sulphonamides are so often prescribed for the treatment of gram-negative bacterial infections and emergence of bacterial resistance to antibiotics of these classes would be a very serious warning to both Medical and Veterinary practitioners

all over the world (Suleiman *et al.*, 2014). Antimicrobial resistances among *E. coli* infected patients are globally on the increase and these have been associated with the emergence and spread of ESBL, prolonged and indiscriminate use of antibiotics which has swung several researchers in to action. The current study revealed that out of the 111 *E. coli* isolates tested, 91 (82.0%) exhibited multidrug resistance (MDR). Adding the MDR-*E. coli* isolates resistance to 4 and 3 classes of antibiotics together on table 3 in relation to sample source, will reveal that out of the 91 (82.0%) MDR-*E. coli* isolates, 51 (56.0%) were from cattle, 23 (25.3%) were from sheep and 17(18.7%) were from herders respectively. This overall high prevalence of MDR *E. coli* (82.0%) is consistent with other findings globally; although, the exact proportions and sources may vary depending on the geographical location, type of animals husbandry practices and pattern of antibiotics usage. Several studies all over the World have also reported high prevalence of MDR *E. coli* in cattle, supporting the findings of the current study. Wong *et al.* (2019) reported overall MDR- *E. coli* isolates rate of 75% from cattle in the United States which were resistance to at least three (3) classes of antibiotics and this aligns closely with the 82.0% MDR rate found in this study. In another study conducted by Tadesse *et al.* (2019) in Ethiopia, also reported high MDR rate of 70% in cattle and 60% in sheep, which reflect again a similar trend of high MDR rate in cattle and sheep. These also suggest that the MDR *E. coli* rate is on the increase globally. The 18.7% MDR isolates from herders suggest possible zoonosis between livestock and humans. Liu *et al.* (2020), reported MDR *E. coli* colonization rate of 20% in farm workers in China which is comparable with the 18.7% from herders in this study. However, the high ratio of 56.0% :

25.3% MDR rate from cattle to sheep is consistent with the report of Nguyen *et al.* (2016) who reported MDR – *E. coli* ratio of 60% : 30% cattle to sheep in Vietnam.

In contrast, Abraham *et al.* (2021), reported MDR *E. coli* rate of only 40% in cattle in part of Europe which may possibly due to strict/effective antimicrobial stewardship policies.

However, an estimate of 58,000 neonatal septic deaths was attributed to drug-resistance infections in India alone (Laxminarayan *et al.*, 2015). The United States Centres for Disease and Prevention (CDC) estimated that antibiotics resistance is responsible for more than 2 million infections and 23,000 deaths each year in U.S. (CDC, 2013). In Europe an estimate of 25,000 deaths is attributed to antibiotic-resistant infections (EMA and CDC, 2009).

Phenotypic Screening of MDR- *E. coli* Isolates from Livestock and Herders Based on Detection Methods in this Study

Out of the 91(100%) MDR *E. coli* isolates subjected to ESBL production tests using the phenotypic methods of Double Disc Synergy Test (DDST) and Disc Replacement Method (DRM), the highest ESBL producers (37) were detected by DDST. Out the 37 ESBL Producers, 21(20.6%) were from cattle, 09(8.8%) and 07(6.9%) were from sheep and herders respectively.

Using the DRM phenotypic screening tests out of the 91(100%) *E. coli* isolates tested for ESBL production, a total of twenty (20) ESBL producers were detected. Of the 20 ESBL producers, 10 (9.8%) were from cattle, 05 (4.9%) were from sheep and 05 (4.9%) were from the herders isolates respectively (Table 4)

Table 4: Distribution of ESBL-*E. coli* Isolates from Livestock and Herders Based on Detection Methods in this Study

Sample source	MDR <i>E. coli</i> isolates (n=91)	No. (%) of ESBL- <i>E. coli</i> isolates based on methods of detection	
		DDST (n=37)	DRM (n=20)
Cattle	51	21(23.1)	10(1.9)
Sheep	23	09(9.9)	05(5.5)
Herders	17	07(7.7)	05(5.5)

The prevalence of ESBL producing genotypes among *Enterobacteriaceae* including *E. coli* is rapidly on the increase and vary greatly throughout the world. This rapid increase in prevalence calls for reliable laboratory screening methods which will identify the presence or absence of these ESBL enzymes in clinical isolates (Bradford, 2001). However, various susceptibility testing methods differ in their ability to detect MDR-ESBL producing *E. coli* isolates. In the course of ESBL detection by several screening methods, some ESBL producing isolates may test falsely positive or falsely negative especially when compared to the gold standard method such as multiplex-PCR.

In this study, the two most widely used DDST and DRM were employed for use with view to comparing their prevalence, sensitivities and specificities at the screening levels. Phenotypic screening for ESBL producing bacteria is a broad based detection method which identifies all resistant factors such as plasmid mediated ESBL, AmpC-beta-lactamases, chromosomal ESBL, hypermucoviscosity and carbapenemases. ESBL detection involves two important steps. Firstly, an indicator test with antibiotics looking for multidrug resistance (MDR) or reduced susceptibility to identify isolate's likelihood to harbouring ESBL. Secondly, to detect the synergy between an oxyimino-cephalosporin and clavulanic acid, distinguishing isolates with ESBL from those

that are resistant due to other factors (Gay *et al.*, 2018). In this study, out of the 91(100%) MDR *E. coli* isolates subjected to ESBL production tests using the Double Disc Synergy Test (DDST) and Disc Replacement Method (DRM), the highest ESBL producers (37) were detected by DDST presenting a prevalence of 40.7% (37/91). Out the 37 ESBL Producers, 21 (23.1%) were from cattle, 09 (9.9%) were from sheep and 07 (7.7%) were from herders respectively.

Using the DRM phenotypic screening tests, out of the 91(100%) *E. coli* isolates tested for ESBL producers in this study, a total of twenty (20) ESBL producers were detected thereby presenting a prevalence of 21.9% (20/91). Of the 20 ESBL producers, 10 (10.9%) were from cattle and 05 (5.5%) each were from sheep and herders isolates respectively In previous studies, Giriyaapur *et al.* (2011) reported the prevalence of 63.9%; Singal *et al.* (2005) reported a prevalence of 63.6%. Ravi *et al.* (2011) on the other hand reported a prevalence of 56.2% ESBL producing phenotypes among gram-negative bacilli with inter-disc distance of 20mm using DDST which are all higher than the 36.3% obtained in this study. Disc replacement method (DRM) detected a prevalence of 21.9% (20/91) which was relatively lower than the 31.7% (52/164) detected by Garba and Yushau, (2012). In this study, Cattle on one hand accounted for the highest prevalence rate of 23.1% as against 9.9% and 7.7% of

sheep and herders respectively. This suggests that cattle are likely to be more vulnerable to infection by ESBL producing *E. coli* pathogens.

Comparison of the Prevalence, Sensitivity and Specificity Values, in Relation to Methods of Detection in this Study

In this study, Double Disc Synergy Test (DDST) and Disc Replacement Method (DRM) were used simultaneously for phenotypic screening and multiplex-PCR (M-PCR) was used

as diagnostics or gold standard test for the comparison of the sensitivity and specificity of the two phenotypic screening methods and their sensitivities and specificities were calculated. Out of the 91 MDR *E. coli* isolates subjected to screening by DDST and DRM protocols, the sensitivity of 100% and specificity of 67.6% were due to DDST protocol while the sensitivity of 57.1% and specificity of 42.9% were due to DRM respectively (Table 5).

Table 5: Distribution of Sensitivity and Specificity Values in Relation to Detection Methods Used in this Study

Phenotypic methods	% Sensitivity	% Specificity	Remarks
DDST	100	67.6	High sensitivity, moderate specificity
DRM	57.1	42.9	Low sensitivity, low specificity

The results of the two phenotypic methods used in the current study can be evaluated and compared as to which of these two screening methods is more sensitive and specific. This can be possible after when the diagnostic or gold standard test result is obtained. In this study, molecular methods of Multiplex-PCR was used as the diagnostic test and hence, recorded the sensitivity and specificity 100% respectively and its result was used as the gold standard for comparison.

Sensitivity of any given test is its ability to detect all the true-positive cases while specificity is its ability to detect all the true-negative cases correctly (Khan *et al.*, 2022).

In this study, DDST protocol recorded a sensitivity of 100% which suggests that all positive cases were detected thereby making it highly reliable for screening purpose. However, its specificity of 67.6% suggests that about one-third non-ESBL producers were falsely identified as ESBL producers which may lead to unnecessary treatments or further confirmatory testings. These values indicate that DDST has a substantially higher sensitivity and specificity compared to DRM for detecting ESBL production in *E. coli* isolates of livestock and herders in north eastern States of Nigeria. Sensitivity (true positive rate) of 100% recorded by DDST in this study means it has correctly identifies all ESBL producing isolates thereby minimizing false negative rate. These findings align with several recent studies that have evaluated the use of DDST in detecting ESBL production. For instance, a study by Paterson and Bonomo (2020) underscored the fact that DDST remains a widely accepted phenotypic method with high sensitivity or ESBL detection, although specificity can be variable depending on the bacterial species and antibiotic disc used. Similarly, in a recent study by Khan *et al* (2022) on ESBL producing *E. coli* isolates from livestock reported DDST sensitivity ranging from 90-100% which corroborates well with the current findings. Many recent studies have consistently shown that DDST is a reliable phenotypic method for ESBL detection with high sensitivity and moderate specificity. Paterson and Bonomo (2019) highlighted DDST remains a standard phenotypic confirmatory test due to its high sensitivity and simplicity of performing in resource-limited settings. Tamang *et al.* (2022) compared various phenotypic methods and found that DDST exhibited superior sensitivity approaching 100% as compared to other disc-based methods including DRM. Vercaileren *et al.* (1997) reported the sensitivities of 96.9% and 90.6% with DDST and three-dimensional tests respectively. Giriya *et al.* (2011) reported sensitivity range from 79.8% to 89.5% in different studies using DDST Protocol which are all lower than the sensitivities recorded in this study. This may be due to variation in the inter-disc distance, difference in sample sizes and/or concentrations of the antibiotics used.

Relatively lower specificity by DDST test in this study might be due to the presence of other beta-lactamases such as AmpC or methodological differences, including disc placement distance, or incubation condition. Moreover, the co-existence of multiple resistance factors in *E. coli* isolates from animals and humans could complicate phenotypic detection, which may lead to false positives (Paterson and Bonomo, 2020).

The DRM protocol recorded a sensitivity and specificity of 57.1% and 42.9% respectively which is lower than those presented by DDST method and these indicate poorer performance in correctly detecting both the ESBL, and non-ESBL producers. DRM scoring the sensitivity of 57.1% means the protocol has misses out a significant number of ESBL producers thereby maximizing the false negative rate. On the other hand, specificity (true negative rate), DRM was also outperformed by DDST in specificity by correctly identifying non-ESBL producers at the rate of 42.9% against the 67.6% for DDST which suggest that DDST produces lesser false negatives than DRM. The low sensitivity and specificity observed in this study may be attributed to methodological variability and / or interpretation challenges associated with DRM as was also observed by Sigh *et al* (2021) who reported suboptimal performance of DRM as compared to DDST and other confirmatory methods such as combine Disc Test (CDT).

The findings of this study are consistent with reports of other recent previous researchers in the literatures. For example Lee *et al.* (2023) reported DRM sensitivity to be around 60-65% and specificity below 50%. Based on the sensitivity and specificity values recorded in this study, DDST appeared to be the most sensitive and specific phenotypic method for detecting ESBL production in *E. coli* isolates among the two methods evaluated.

Sensitivity and positive predictive value (PPV) of a test method could be able to determine the accuracy of such test to detect all positive cases. While specificity and negative predictive value (NPV) of a test method should be able to identify all negative cases. In a particular test, as the sensitivity / PPV increase, the specificity / NPV tend to decrease. As sensitivity / PPV increase toward 100%, the test-method approaches gold standard (Alireza *et al.*, 2015). However, the sensitivity of 100% and specificity of 67.6% obtained in this study at single 20mm inter-disc distance, suggests that DDST method is most sensitive and moderately specific as compared to the DRM protocol. Therefore, DDST can stand alone in effective ESBL phenotypic screenings. Nevertheless, when the 36 ESBL producing *E. coli* phenotypes were subjected to molecular confirmation by multiplex-PCR in which both sensitivity and specificity were 100%, DDST tends to be more sensitive but less specific whereas DRM was moderately sensitive and less specific.

Statistical analysis showed significant difference regarding the two methods of phenotypic screening (P -Value < 0.05). Generally, in the current study about 65.0% were either false positive, or resistant due to other forms of resistant factors such as Carbapenemase, (*Bla* OXA, PER, VEB, TLA, BEL, NIM etc.), chromosomal ESBL (AmpC), and/or Hypermucoviscosity which were not tested in this analysis. However, more comprehensive study involving primers of wide spread resistant factors, covering larger sample size is required to ascertain the exact sensitivity and specificity of various methods of ESBLs detection.

CONCLUSION

In conclusion, it is important to note that phenotypic screening methods are broad-based tests which detect plasmid mediated ESBL resistance and resistance due to other forms of resistant factors such as carbapenemase, (*Bla* OXA, PER, VEB, TLA, BEL, NIM), chromosomal ESBL (AmpC), and/or hypermucoviscosity. The sensitivity of 100% (DDST)/ 57.1% (DRM) and specificity of 67.6% (DDST) / 42.9% (DRM) were obtained in this study suggests that DDST method is the most sensitive and moderately specific as compared to the DRM protocol. This study revealed significant difference regarding the sensitivity and specificity of the two methods of phenotypic screening (P -Value < 0.05). Therefore, for reliable, sensitive and specific phenotypic screening results, DDST is more preferable over the DRM protocol.

ETHICAL CONSIDERATION

Ethical clearance certificate was obtained before embarking on sample collection from live animals.

CONFLICTS OF INTEREST

The authors in this study declare that they do not have any conflicting interest

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