International Journal of Pharmaceutical Science Invention
ISSN (Online): 2319 – 6718, ISSN (Print): 2319 – 670X
www.ijpsi.org Volume 2 Issue 3 ǁ March 2013 ǁ PP.38-44

Antimicrobial Resistance Patterns Of *Escherichia Coli* O157:H7 From Nigerian Fermented Milk Samples In Nasarawa State, Nigeria.

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ABSTRACT: *Escherichia coli* O157:H7 is an emerging public health concern in most countries of the world. It is an important cause of food-borne human disease. This study determined the antibiotic resistance patterns of *E. coli* O157:H7 from Nigerian fermented milk consumed in Nasarawa State, Nigeria. A total of 420 locally-fermented milk samples were obtained for this study. The samples were bacteriologically analyzed in the laboratory for the presence of *E. coli* O157:H7 by means of cultural techniques (enrichment on modified tryptone soy broth and selective plating on Cefixime-Tellurite Sorbitol Mac-Conkey Agar), biochemical (Microbact 12E) and serological assays. Oxoid diagnostic kit; latex (R30959601) was used to confirm *E. coli* O157:H7 respectively. Confirmed isolates were further subjected to antimicrobial susceptibility test using the Agar disc diffusion technique. The results of the study showed that out of 420 locally-fermented samples examined, 19 (4.5%) were contaminated with *E. coli* O157:H7. Among samples examined, the highest occurrence rate (5.7%) was recorded in samples obtained from Akwanga, Wamba and Doma Local Government Areas while Lafia and Keffi had the least occurrence rate (2.9%). Antibiotics susceptibility profile showed that all the isolates were resistant to multiple antibiotics, except ciprofloxacin and gentamicin, resulting in nine different resistance patterns. All the nineteen (100%) of the isolates were resistant to penicillin and tetracycline, 18 (94.7%) to erythromycin, 16 (84.2%) to amoxicillin, oxacillin and sulphamethoxazole/trimethoprim, 13 (68.4%) to chloramphenicol and 8 (42.1%) to streptomycin. 15 (78.9%) and 17 (89.5%) of the isolates were sensitive to ciprofloxacin and gentamicin. The predominant antimicrobial resistance pattern was penicillin-tetracycline-chloramphenicol-amoxycilin-erythromycin-oxacillin-sulphamethoxazole/ trimethoprim with the occurrence rate of 36.8% from samples obtained from Wamba, Doma, Kokona and Keffi Local Government Areas. The consumption locally-fermented milk has potential health risks to consumers in Nasarawa State, hence proper hygiene in the process-line and marketing of this product is recommended. The multiple antimicrobial resistance exhibited by *E. coli* O157:H7 strains in this study is an indication of possible antibiotic abuse.

Keywords: *E. coli* O157:H7, emerging, locally fermented milk, Antibiotics, resistance.

I. INTRODUCTION

Escherichia coli O157:H7 is an emerging public health concern in most countries of the world (Schlundt 2001). It is an important cause of foodborne human disease. Complications related to infection include diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (Nataro and Kaper, 1998).Since the first reported case of *Escherichia coli* O157:H7 in 1982 in USA, outbreaks and sporadic cases of disease due to this organism have also had a fairly wide geographic distribution in the Africa continent (Isibor *et al*., 2012). Several outbreaks of E.coli O157 have been epidemiologically attributed to consumption of contaminated fresh and dry sausage and other meat products (Chinen *et al*., 2001). Other foodstuffs including unpasteurized milk and dairy products manufactured from raw milk, however, have been implicated in many outbreaks, (Maher *et al*., 2001).
Although antibiotics are not recommended for treatment of *E. coli* O157:H7 infections in humans, there is evidence that bacterial isolates are resistant to some antibiotics (Aibinu *et al*., 2007). The extensive use of antibiotics in both human medicine and for agricultural purposes, particularly, in disease prevention and growth promotion in animal production is a considerable cause of the selection and prevalence of antibiotic resistant *E. coli* O157:H7 (Schroeder *et al.*, 2002; Callaway *et al*., 2003). The development of resistance to antimicrobials is known to occur through stable genetic change heritable from generation to generation through specific mechanisms including mutation, transduction, transformation and or conjugation (Goodman *et al*., 1990).
Because some antibiotics may cause bacterial lysis and liberate the free Shiga toxins in the intestinal tract (Karch *et al*., 1987; Wong *et al*., 2000), the antimicrobial treatment is contraindicated for human *E. coli*

O157:H7 infections. However, such treatments may be recommended for cystitis and pyelonephritis other than haemorrhagic colitis all caused by *E. coli* O157:H7 (Griffin, 1991). For those limitations of using antimicrobial agents in *E. coli* O157:H7 cases, the generally accepted belief is that the *E. coli* O157:H7 may still be susceptible to most antimicrobials. In addition to their epidemiological importance, the studies of antimicrobials susceptibility of *E. coli* O157:H7 may have more therapeutic significance as recent studies have indicated a possible role of early administration of antimicrobials in preventing the progression of haemolytic uremic syndrome and haemorrhagic colitis both caused by *E. coli* O157:H7 (Molbak *et al*., 2002). The aim of this study was to determine the antibiotic resistance patterns of *E. coli* O157:H7 from Nigerian fermented milk consumed in Nasarawa state, Nigeria.

II. MATERIALS AND METHODS

Sample Collection and Handling

Four hundred and twenty (420) locally-fermented milk (*nono*) samples were obtained across Nasarawa State, Nigeria between April to November, 2012 for this study. Seventy (70) *nono* samples were randomly purchased from *nono* hawkers in each of the Local Government Area selected for this study viz: Akwanga and Wamba (Nasarawa North), Lafia and Doma (Nasarawa South) and Keffi and Kokona (Nasarawa West) respectively. Convenient sampling method was used for sampling. The selection of these areas was based on cattle population and *nono* hawkers. Each sample (25ml) was collected into a sterile corked plastic tube and then labelled appropriately. All samples were placed in separate sterile plastic bags to prevent spilling and cross contamination. Samples were stored in a cooler with ice packs and then transported to the Bacterial Zoonoses Laboratory of the Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University Zaria, for Laboratory analysis within 4 to 5hours.

Isolation of *E. coli* O157:H7

The steps for the isolation of *E. coli* O157:H7 were conducted according to the isolation procedure of ISO, (2003). These steps included: Enrichment, Selective plating, biochemical characterization and serological confirmation by latex agglutination.

Enrichment of the Locally-Fermented Milk (*nono*) Samples

25ml of the *nono* sample was directly added to 225ml modified Tryptone soy broth supplemented by novobiocin (mTSB+N). The inoculated broths were incubated at 37^{o}C for 24hours.

Selective Plating and Identification of *E. coli* O157:H7 Colonies

A loopful of the enriched sample (inoculated broth) was streaked onto Telluritte-Cefixime Sorbitol MacConkey agar (CT-SMAC agar) plate and incubated at 37^{o}C for 24 hours.

A typical *E. coli* O157:H7 appeared as a non-sorbitol fermenter colony (NSFC) which is characterized as having a slightly transparent, almost colourless with a weak pale brownish appearance. About 4 to 5 colonies from the CT-SMAC agar plates were picked and sub-cultured onto non-selective medium, such as nutrient agar slants at 37^{o}C for 24hours, and refrigerated for further biochemical and serological analysis. Also, NSFC were examined for the presence of gram negative rods by gram staining.

Biochemical Test

The strains were characterized biochemically using Microbact 12E (MB1130A^{+}, Oxoid) according to the manufacturer's instruction. Identification of *E. coli* strains was done following a series of 12 biochemical tests.

Serological Test

E. coli positive colonies were serologically confirmed by using *E. coli* O157:H7 latex agglutinations assay (R30959601, Oxoid), containing latex particles coated with antibodies specific for *E. coli* O157 and *E. coli* H7 antigen. Identification of *E. coli* O157:H7 was carried out following the manufacturer's instruction, hence colonies that agglutinated were considered to be *E. coli* O157:H7.

Antimicrobial Susceptibility Test

The isolates were screened for antimicrobial susceptibility, using the agar disk diffusion method by Kirby-Bauer, (1966). The following antibiotics (Oxoid) were used: Penicillin (10 units), Gentimicin (10µg), Ciprofloxacin (5µg), Streptomycin (10µg), Amoxycillin (25µg), Tetracycline (30µg), Chloramphenicol (30µg), Oxacillin (5µg), Erythromycin (5µg) and Sulphamethoxazole/Trimethoprim (25µg).

The isolates were uniformly streaked on Muller-Hinton agar plate and the antibiotic impregnated discs were applied onto the inoculated plates using sterile forceps. The plates were then incubated at 37^{o}C for 24hrs, after which clear zones of inhibition for each antibiotic were measured using transparent ruler. The results were interpreted using the Clinical and Laboratory Standards Institute (CLSI) criteria (CLSI, 2007).

III. RESULTS

Prevalence of *E. coli* O157:H7 and Antimicrobial Susceptibility of Isolates

Table 1 summarizes the results of the occurrence of *E. coli* O157:H7 in fermented milk (*nono*) samples obtained from various Local Government Areas in Nasarawa State. Out of the 420 *nono* samples examined, 19 (4.5%) were found to be contaminated with *E. coli* O157:H7. The results also indicated the percentages of the occurrence of the pathogen across the selected Local Government Areas in Nasarawa States viz: Akwanga 4 (5.7%), Wamba 4 (5.7%), Lafia 2 (2.9%), Doma 4 (5.7%), Keffi 2 (2.9%) and Kokona 3 (4.3%) respectively

Table 2 shows the antimicrobial susceptibility patterns of the 19 *E. coli* O157:H7, isolated from locally fermented milk (*nono*) samples, using 10 antibiotics. 17 (89.5%) of the isolates were sensitive to gentamicin, 15 (78.9%) were sensitive to ciprofloxacin, 8 (42.1%) were sensitive to streptomycin, 3 (15.8%) were sensitive to oxacillin and sulphamethoxazole/trimethoprim, 1 (5.3%) was sensitive to chloramphenicol and amoxicillin and none was sensitive to penicillin, tetracycline and erythromycin respectively.

Antibiotic susceptibility profile showed that virtually all the isolates were resistant to one or multiple antibiotics. However, 19 (100%) of the 19 *E. coli* O157:H7 isolates were resistant to penicillin and tetracycline, 18 (94.7%) were resistant to erythromycin, 16 (84.2%) were resistant to amoxicillin, oxacillin and sulphamethoxazole/trimethoprim, 13 (68.4%) were resistant to chloramphenicol, 8 (42.1%) were resistant to streptomycin and none was resistant to ciprofloxacin and gentamicin (Table 2). Gentamicin, ciprofloxacin and streptomycin were the most sensitive antibiotics whereas penicillin, tetracycline and erythromycin were the least sensitive. The total percentage resistivity of the antibiotics tested against the *E. coli* O157:H7 isolates was 51.3% (Table 3).

The antimicrobial resistance patterns are shown in Table 4. The most common patterns were penicillin-tetracycline-chloramphenicol-amoxycillin-erythromycin-oxacillin- sulphamethoxazole/trimethoprim 7 (36.8%), penicillin-streptomycin-tetracycline-chloramphenicol-amoxycillin-erythromycin-oxacillin-sulphamethoxazole/trimethoprim 3 (10.5%), penicillin-strepromycin-tetracycline-amoxycillin-erythromycin-oxacillin-sulphamethoxazole/trimethoprim 2 (10.5%) and penicillin-tetracycline-amoxycillin-erythromycin-oxacillin-sulphamethoxazole/trimethoprim 2 (10.5%). Penicillin and tetracycline resistance were most common among the various patterns observed. The highest levels of multidrug resistance observed were in isolates from Wamba, Doma, Kokona and Keffi Local Government Areas respectively. The relationship between the patterns of antibiotic resistance and number of isolates showing such pattern is presented in Table 5.

Table 1. Prevalence of *E. coli* O157: H7 in Locally-Fermented Milk (*Nono*) Sold in Nasarawa State, Nigeria.

LGA	No. (%) of Samples	No. (%) Positives
Akwanga	**70 (16.7)**	**4 (5.7)**
Wamba	**70 (16.7)**	**4 (5.7)**
Lafia	**70 (16.7)**	**2 (2.9)**
Doma	**70 (16.7)**	**4 (5.7)**
Keffi	**70 (16.7)**	**2 (2.9)**
Kokona	**70 (16.7)**	**3 (4.3)**
Total	**420 (100)**	**19 (4.5)**

Table 2. Antimicrobial Susceptibility Pattern of *E .coli* O157:H7 Isolates from Locally Fermented Milk (*Nono*) Sold in Nasarawa State.

		Susceptibility		
		R	I	S
Antibiotic	Concentration (µg)	No. (%)	No. (%)	No. (%)
Penicillin	*10*	*19 (100.0)*	*0 (0.0)*	*0 (0.0)*
Ciprofloxacin	5	0 (0.0)	4 (21.1)	15 (78.9)
Gentamicin	10	0 (0.0)	2 (10.5)	17 (89.5)
Streptomycin	10	8 (42.1)	3 (15.8)	8 (42.1)
Tetracycline	30	19 (100.0)	0 (0.0)	0 (0.0)
Chloramphenicol	30	13 (68.4)	5 (26.3)	1 (5.3)
Amoxicillin	25	16 (84.2)	2 (10.5)	1 (5.3)
Erythromycin	5	18 (94.7)	1 (5.3)	0 (0.0)
Oxacillin	5	16 (84.2)	0 (0.0)	3 (15.8)
sulphamethoxazole/ trimethoprim	25	16 (84.2)	0 (0.0)	3 (15.8)

Table 3. Percentage Resistivity of the Tested Antibiotics Against *E. coli* O157:H7 Isolates from Nasarawa State.

LGA	No. of Isolates	No. (%) of Antibiotic Resistant
Akwanga	4	26(65)
Wamba	4	24 (60)
Lafia	2	13 (32.5)
Doma	4	29 (72.5)
Keffi	2	12 (30)
Kokona	3	19 (47.5)
Total	19	123 (51.3)

Table 4. Antimicrobial resistance Patterns of 19 Isolates of *E. coli* O157:H7 from Locally Fermented Milk (*nono*) Sold in Nasarawa State.

No. of Antibiotics	Resistance Pattern	No. (%) of Isolates	LGA
4	Pen, Tet, Chl, Ery	1 (5.3)	Wa
4	Pen, Str, Tet, Sul	1 (5.3)	Ak
5	Pen, Str, Tet, Ery, Sul	1 (5.3)	La
6	Pen, Tet, Amo, Ery,Oxa, Sul	2 (10.5)	Ke, Ko
6	Pen, Tet,Chl, Amo, Ery,Oxa	1 (5.3)	Wa
7	Pen, Tet, Chl, Amo, Ery, Oxa, Sul	7 (36.8)	Wm, Do, ko, ko
7	Pen, Str, Tet, Amo, Ery, Oxa, Sul	2 (10.5)	Do, Ak
7	Pen, Str, Tet, Chl, Amo, Ery, Oxy	1 (5.3)	Ak
8	Pen, Str, Tet, Chl, Amo, Ery,Oxa, Sul	3 (15.8)	Do, Ak, La

Symbols: Pen, penicillin; Tet, tetracycline; Chl, chloramphenicol; Cip, ciprofloxacin; Gen, gentamicin; Str, streptomycin; Ery, Erythromycin; Amo, Amoxycillin; Oxa, oxacillin; Sul, sulphamethoxazole/trimethoprim; Wa, Wamba; Ak, Akwanga; La, Lafia; Ke, Keffi; Ko, Kokona and Do, Doma.

Table 5. The Relationship Between the Patterns of Antibiotic Resistance and Number of Isolates

S/N	No. of Antibiotic	No. (%) of Isolates
1	**4**	**1 (5.3)**
2	**4**	**1 (5.3)**
3	**5**	**1 (5.3)**
4	**6**	**2 (10.5)**
5	**6**	**1 (5.3)**
6	**7**	**7 (36.8)**
7	**7**	**2 (10.5)**
8	**7**	**1 (5.3)**
9	**8**	**3 (15.8)**

IV. DISCUSSION

The prevalence of *E. coli* O157:H7 in locally-fermented milk (*nono*) in Nasarawa State recorded in this study is 4.5%. This is higher than the data reported in Plateau State, Nigeria, where 5 (0.71%) out of 350 *nono* samples and 21 (3.00%) from 350 raw milk samples were detected (Itelima *et al*., 2010). The occurrence of this organism in this study is also higher than the data reported in raw milk from the United States of America in which out of the 1, 021 bovine milk samples examined, 20 (2.0%) were positive for *E. coli* O157:H7 (Armstrong *et al*., 1996). Lower occurrence rate of *E. coli* O157:H7 (O.5%) in traditional dairy products was recorded from a study in Iran (Rahimi *et al*., 2012) as compared with the result obtained from this study.

E. coli O157:H7 has also been isolated from yoghurt which is also a fermented milk product (Morgan *et al*., 1993). These authors have reported that the occurrence rate was as high as 5% in some yoghurt samples and some samples contained the organism at levels of ≤ 10 cells per millilitre contamination. They suspected that contamination of fermented milk samples may have come from raw milk used for its production or from handlers due to poor processing. The significance of the isolation of *E. coli* O157:H7 from this local milk product (*nono*) is of general public health concern because many people in Nigeria, mostly in the northern States consume this product. Thus, the product may be one of the major vehicles for *E. coli* O157:H7 transmission from cattle to man.

The relatively higher occurrence of *E. coli* O157:H7 in locally-fermented milk (*nono*) in Nasarawa State, Nigeria may be attributed to lack of effective sanitary precautions and less careful handling procedures during milking process and *nono* production. The use of traditional milking methods also expose milk to pathogenic bacteria found in cow udders and probably on the hands of the milkers who may have come in contact with faeces of the cows. The unhygienic environmental conditions where *nono* is marketed may also contribute to its contamination. The use of more sensitive assay such as enrichment broth and selective media for the isolation of *E. coli* O157:H7 in this study might have helped in obtaining a higher occurrence of the organism. In a study in USA by Clark *et al*. (1989), *E. coli* O157:H7 was not isolated from any of the milk samples examined. These authors suggested that the absence of the organism in milk was as a result of the use of assay which was insensitive to *E. coli* O157:H7.

The antimicrobial sensitivity tests showed a high level of resistance to most of the antibiotics used. The development of antimicrobial resistance by the bacteria to these drugs poses a major challenge in both human and animal medicine because these drugs are commonly used in the treatment of human patients and in veterinary practice. Uncontrolled usage of antibiotics in treatment of animals and their incorporation in animal feeds has been suspected to account significantly to the increase in antibiotic resistance in human bacterial isolates (WHO, 2000; Galland *et al*., 2001).

Penicillin and tetracycline resistance were the highest. All the isolates (100%) tested were resistant to both antibiotics. This is in agreement with the finding of Olatoye (2010), which recorded a high level of tetracycline resistance of 91.4% among isolates of *E. coli* O157:H7. Al Haj *et al.* (2007) also observed high resistance to tetracycline (81.4 %); Shitandi and Sternesjö (2001) obtained also high resistance to penicillin (72%) and to Tetracycline (57.9%); O'Brien (1987) also reported high resistance to tetracycline (72%). The high level of resistance of tetracycline obtained in this study may be as a result of it being the most commonly available antibiotic used as growth promoter and routine chemoprophylaxis among livestock in Nigeria (Olatoye, 2010). This is worrisome considering that tetracycline is a first line drug in Nigeria, and as in most developing countries, people with gastrointestinal infections readily purchase it across the counter for self-medication (Chigor *et al*., 2010). Penicillin resistance as obtained from this study may be as a result of the frequent usage of this antibiotic in treating diseases in cattle (Byrne *et al*., 2003). According to Hart and Kariuki (1998) and Okeke *et al*. (1995) penicillin and tetracycline are known to be extensively used in developing countries.

High rate of resistance to sulphamethoxazole/trimethoprim (84.2%) is in agreement with the previous work by Shroeder *et al*. (2001), who reported that among 189 *E. coli* O157:H7 isolates recovered from various sources between 1985 and 2000, 19 (10%) were resistant to this antibiotic. This antimicrobial is commonly used to treat respiratory infections, diarrhoea, mastitis, and other infections in beef and dairy cattle. Resistance was found to be relatively low in streptomycin. This probably may be because of less exposure to the antibiotic due to the discourage use of the antibiotic and the fact that it is usually administered intravenously thereby restricting indiscriminate use (Cheesbrough, 2000). This shows that streptomycin can be used as an antibiotic of choice against *E. coli* O157:H7 infections, except for its serious side effect (Prescott *et al*., 2005)

The high prevalence of resistance of *E. coli* O157:H7 isolates to erythromycin, oxacillin, amoxicillin and chloramphenicol is of importance from the view point of medical and veterinary practice in Nigeria. This could be a reflection of use and misuse of these antibiotics in the society. This finding is not surprising because outside the hospital environment the general population have easy access to various antibiotics at any drug store without any prescription from a medical practitioner.

In this study, all the *E. coli* O157:H7 isolates tested showed multidrug resistance to the antibiotics tested at various percentages. This result is in agreement with the findings by other researchers, who reported multidrug resistance among *E. coli* O157:H7 isolates (Kim *et al*., 1994; Schroeder *et al*., 2002**).** Various isolates were resistant to 4, 5, 6, 7 and 8 of the antibiotics tested. Isolates from Doma, Akwanga and Lafia showed higher frequencies of multidrug resistance. Multiple antimicrobial resistance in *E. coli* O157:H7 isolates may partly result from the spread of genetic elements including plasmids, transposons, and integrons (Zhoa *et al*., 2001) that may confer resistance to numerous antimicrobials. According to Aarestrup (1995) and Levin *et al*. (1997), multiple resistances capable of regional dissemination can emerge as a result of antimicrobial selection pressure in either livestock or humans. Evidence has been found which indicates that resistant strains of pathogens can be transmitted to humans through food (Oosterom, 1991; Khachatourians, 1998).

This increase in multidrug resistance is quite alarming coupled with the fact that all these isolates harboured plasmids on which these genes may be located and which are highly transferable. The selection and spread of resistant organisms in developing countries, which can often be traced to complex socioeconomic and behavioural antecedents, has contributed to the escalating problem of antibiotic resistance worldwide (Okeke *et al*., 1999).

Results from this study indicate that ciprofloxacin (floroquinolone) and gentamicin (aminoglycoside) are the drugs of choice for *E. coli* O157:H7, since none of the isolates was resistant to them. This shows the

effectiveness of the floroquinolones and aminoglycosides, and is in agreement with the finding of Scheld, 2003, suggesting the use of this class of antibiotics.

The public health significance of these findings is that antimicrobial resistant *E. coli* O157:H7 from *nono* (or dairy animals) may colonize the human population via the food chain, consumption of *nono*, contact through occupational exposure, or waste runoff from *nono* production facilities to the neighbourhood. Indiscriminate use of antimicrobials among livestock producers and marketers in Nigeria could also be responsible for the resistance pattern obtained in this study.

The development of antimicrobial resistance by the *E. coli* O157:H7 isolates to these drugs poses a major challenge in both human and animal medicine because these drugs are commonly used in the treatment of human patients and in veterinary practice. Uncontrolled usage of antibiotics in treatment of animals and their incorporation in animal feeds has been suspected to account for majority of the increase in antibiotic resistance in human bacterial isolates (WHO, 2000; Galland *et al*., 2001). The developmental of resistance to antimicrobials occurs through stable genetic change heritable from generation to generation through specific mechanisms including mutation, transduction, transformation, and or conjugation (Goodman *et al*., 1990; Metlay *et al*., 2006). The shedding of the resistant bacteria into the environment by cattle may lead to a widespread dissemination of antibiotic resistant genes to the resident bacteria in the environment (Callaway *et al*., 2003, 2004; Mashood, *et al*., 2006).

VII. CONCLUSION

The antimicrobial susceptibility of *E. coli* O157:H7 isolates showed a high prevalence of resistance to most of the antibiotics used. The data suggest that selection pressure imposed by the use of these antibiotics whether therapeutically in human and veterinary medicine or as prophylaxis in the animal production, is a key driving force in the selection of antimicrobial resistance in *E. coli* O157:H7. There is a need to legislate and enforce laws to limit the prescription and dispensing of antibiotics and other drugs to only qualified professionals. Education of the public on the dangers of indiscriminate purchase and use of drugs is also imperative.

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