OCCURRENCE, ANTIBIOTICS SUSCEPTIBILITY PATTERN, BIOFILM FORMATION AND PHYSIOLOGICAL PROPERTIES OF *ESCHERICHIA COLI* ISOLATED FROM RAW MEAT SAMPLES WITHIN IBADAN NORTH LOCAL GOVERNMENT OF OYO STATE, NIGERIA

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ABSTRACT
Food-borne pathogens are the leading cause of illness and death in developing countries and contaminated raw meat constitutes one main source of food-borne illnesses. Therefore studieswere conducted to investigate the occurrence, antibiotics susceptibility pattern, bio-film formation and physiological properties of Escherichia coli isolated from raw meat samples collected from selectedmarkets within Ibadan North Local Government of Oyo State. Isolation and identification of E.coli species fromraw meat samples werecarried out using the culture dependent method and biochemical characterization respectively while antibiotic sensitivity pattern was studied using thedisc diffusion technique with reference to CLIS guidelines. Bio-film production and physiological properties were investigated based on standard procedures.The result revealed that a sample obtained from retailer 4 in Oje market showed both the highest total bacterial count (TBC) and enterobacterial count (EBC) of 1.46 x 10^5 and1.18 x 10^5cfu/ml respectivelywhile88% produced bio-filmsof which 53%, 26% and 9% are weak, moderate and strong bio-film producers respectively and 12% was non bio-film producers.Only one out of the 73 E.colispp. was hemolytic. The antibiotic sensitivity pattern showed that 98.8% and 83.56% of the E. coli were resistant to ampicillin and gentamycin respectively and 68.48% and 53.42% were susceptible to nitrofurantoin and ofloxacin respectively.In addition, the E. coli spp.grew within the temperature range of 28^0- 40^0C, NaCl concentrations of 2-5% and pH of 4.0-8.0. The high occurrence of E. coli in meat samples is an indication of high level of contamination which might be attributed to poor hygienic conditions of the meathandlers and processing environment.

Key words*:* Occurrence, Eschericia coli, Antibiotic sensitivity pattern, Biofilm, Physiological study

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INTRODUCTION

Meat can bedefined as mammalian flesh with excellent source of protein which is consumed globally and could be eaten alone or as supplementin food product(Duffy, 2006). Nutritionally, it contains 75% water, 19% protein, 2.5% intramuscular fat, 1.2% carbohydrates and 2.3% of other soluble non protein substances, such as amino acids and inorganic substances coupled with minerals (Lawrie and Ledward, 2006). It possesses ahigh water activity of 0.99, which makes it a suitable medium for microbial growth (Subratty and Gurib,2003;Rao *et al.*, 2009) and susceptible to spoilage by microorganisms.The presence of pathogenic microbes in food can lead to food borne infections, resulting inmajor economic losses and threat to human health(Kaferstein, 2003; Komba *et al.*, 2012).The contaminationof meat can be adduced to poor sanitary conditions of slaughter houses and its surrounding environment(Gill *et al.*, 2000; Sachindra*et al.*, 2005; Ercolini *et al.*, 2006). Kozacinski *et al.* (2006) reported that sources of contamination of meat may arise from dirty habits of food-handlers, lack of asepsis at various stages of slaughtering and during transportation of meat and meat products (Schlundt *et al* ., 2004). The production of toxins by these invading microorganisms could be responsible for the food borne diseases they cause. The consumption of meat contaminated with *E.coli*, *Salmonella* spp. *Staphylococcus aureus* and *Listeria monocytogenes*and other pathogenic bacteria have been implicated in food borne

diseases (Vaclavik and Christian, 2008). The high frequency of occurrence of a particular food borne microorganism in meat product is dependent on intrinsic and extrinsic factors of the meat (Ercolini *et al.,* 2006; Li *et al.,* 2006; Adu-Gyamfi *et al.,* 2012). The metabolites producedbypathogenic microorganisms in meat render a change in visual, textural and organoleptic property (Jackson and Mcgowan). However, Doyle (2007)reported that microorganismssuch as *Pseudomonas* spp., *Shewanella*, *Brochothrix*, *Salmonella* spp. *Campylobacter jejuni/coli*, *Yersiniaenterocolitica, E. coli, S. aureus* and *Listeria monocytogenes*grow in meat as food borne pathogens. Yeasts and moldsalsogrow on meat but due to their slow growth, they are not regarded as major component of spoilage microflora. However,*E. coli* and *S. aureus* are normal flora in human and animals but their presence in foods is indication of contamination resulting from excessive human handling (Clarence *et al.,* 2009). From several reports, on food borne pathogens, it could be inferred thatfood contamination with pathogenic *E. coli* constitute up to 25 percent, *Campylobacter jejuni and Shigella*spp. 10-15 percent and 5-15 percent respectively of all diarrheal cases in infants and children *(*Bryan, 1988; Bean and Griffen, 1990). The reported symptoms of food borne illnesses include nausea, vomiting, stomach cramps, mild fever, nausea, vomiting, abdominal pain, diarrhea and in severe cases dehydration and collapse may occur depending on the microorganism causing the illness.The following food products can be a source of transmission of food borne diseases such as potato salad, ham salad, cheeses, cold sweets, dry milk, custards, sandwich, meat, poultry, fish and eggs, milk, soft cheeses, sea foods, bacon sausage, traditionally processed ready-to-eat meat product in Nigeria such as “balangu” (roasted meat), *kilishi, dan-bu-nama, tsire, jirga, ndako, banda, suya* and many more (Yunusa , 2000), beef kebab” in Europe, “kyiskiyma” in central Africa, and “sogodjemine” in Mali (Yusuf *et al.*, 2012).

In the United States, the Centre for Disease Control and Prevention (CDC) (2002)reported that the number of food borne illnesses annually is approximately 76million cases with 325,000 of the reported cases resulting in hospitalization and 5,000 deaths. However, in the United Kingdom, two million cases of food borne illnesses with 3,400 cases occurring in 100,000 inhabitantshad been reported(WHO, 1987).The reported cases of foodborne diseases in both developed and developing countries is approaching an alarming rate, therefore this research work is intended to carry out studies to determine the occurrence, identification, bio-film formation, antibiotics susceptibility pattern and physiological properties of *E. coli* in raw meat samplesand suggesting ways of reducing the incidence of contamination.

MATERIALS AND METHODS

1.Sample Collection

Raw meat samples were purchased from4 retailers in Bodija, Sango, Mokola, Ojee and Gate markets (4 samples from each retailer) located in Ibadan North Local Government area of Oyo state. These samples were kept in sterile nylon bags and transported to the Food and Applied Microbiology laboratory of the department Microbiology of University of Ibadan for further investigation.

2.Bacteriaisolation

The total bacteria and enterobacterial of the meat samples were obtained by plating aliquot of appropriate dilution of the meat extract on nutrient agar and Eosin Methylene Blue agar (EMB) agar respectively and plates were incubated 37^0C for 48h.Distinct representatives colonies were selected and purified by streaking on fresh plates. The pure cultures obtained were stored in glycerol broth(15%) at -15^0C for further use.

3.Identification procedure

The bacterial isolates were identified phenotypically using morphological and biochemical characterizations such as macroscopic andmicroscopicexaminations, Gram staining,Reaction on Triple sugar iron (TSI) agar,Citrate, Indole,Methyl Red (MR),Voges-Proskauer(VP), Catalase Oxidase, Mannitol Sugar, Urease tests and with reference to Bergey's manual of systematic Bacteriology.

4.Pathogenicity Test

4.1.Haemolysis Test

Sterilenutrient agar supplementedwith 5% blood in Petri dishes were inoculated with a loopful of a 24 h old culture and incubated at 37^0C for 24h.Colonies producing clear zones of haemolysis showed positive reaction (Heller and Drabkin, 1979).

4.2.DNase Test

SterileDNAse broth in several Petri dishes were inoculated with a loopful of a 24h old culture and incubated at 37^0C for 24h. The plates were flooded with Hydrochloric acid, formation of clear zones around the colonies indicated positive reaction.

4.3.Gelatinase test

The basal medium used ofcomprised of gelatin (0.4%), yeast extract (0.1%), 1.5% agar. The gelatin was dissolved at 55^0C before addition of the agar and sterilized by autoclaving for 15minutes.Spot inoculation was done on Petri dishes containing the medium and incubated. Zone of clearance around the area of inoculation indicated as positive reaction (Smith and Goodner, 1958).

5.Bio-film Production

One loopful of a 24hold culture was introduced into two ml of Tryptone Soy broth (TSB)in several test tubes and incubated for 48h. The content of the tube was decanted and then washed with Phosphate buffer solution (pH 7.3).Two ml of 4% crystal violet was used to stain the tubes to make the films more visible, excess crystal violet was poured away and the tubes were allowed to dry in an inverted position (Christensen *et al.*, 1982). Bio-film formation was considered positive with the emergence of a visible film lining the wall of the test tube and recorded as 0-absent, 1- weak, 2-moderate and 3-strong.

6.Antibiotics Sensitivity Test

Antimicrobial susceptibility tests were performed by standard disc diffusion technique. Sterile 2ml normal saline in several test tubes was inoculated with one colony from a 24 h old culture and turbidity compared with that of 0.5 McFarland standard and adjusted where necessary. With the aid of sterile cotton swab, the suspension was used to cover the surface of the sterilized Mueller Hinton agar in a Petri dish. The antibiotics disc were placed on it and pressed softly so as to allow contact between the antibiotics disc and the agar and incubated for 24h (Isoken*et al.*, 2013). The zone of inhibition around the disc was measured with the aid of a transparent ruler and then recorded. The results were classified according to CLSI standard 2014.

7.Physiological Studies

7.1.Growth at Different NaClConcentrations

Five ml of sterile Nutrient broth supplemented with 2%, 4% and 5 % sodium chloride (NaCl) was separately dispensed into three different test tubes, inoculated with a loopful of a 24 h old culture and incubated at 37^0C for 24h (Harrigan and McCane, 1966) The optical density was measured using a spectrophotometer set at 560nm.

7.2.Growth at Different Temperature

A loopful of a 24 h old culture was inoculated in sterile Nutrient broth in 3 different test tubes and differently incubated at 28^0C, 37^0C and 40^0C for 24h and the optical density was measured using a spectrophotometer set at 560nm recorded (Harrigan and McCance, 1966).

7.3.Growth at Different pH

Five ml of sterile Nutrient broth was dispensed in 3 separate test tubes and pH adjusted to 4.0, 6.0, 8.0 and inoculated with a loopful of a 24h old culture. Incubation was carried out at 37^0C for24 h. The optical density was measured using a spectrophotometer set at 560nm (Harrigan and McCance, 1976).

RESULTS

The bacterial load of twenty raw meat samples collected from retailers distributed in 5 markets within Ibadan North Local Government, Oyo state, Nigeria is shown in table 1.All the meat samples had bacterial load of varying counts, with a sample obtained from retailer 4 in Oje marketshowing both the highest total bacterial count (TBC) and enterobacterial count(EBC) of1.46 x 10^5 and1.18 x 10^5cfu/ml respectively while a sample obtained from retailer 2 in Sango market had both the least TBC and (EBC) of $6.2x10^1$ and$1.00x10^1$cfu/ml respectively.

Table 1: Total Bacteria Count from meat samples collected from retailers in selected markets located in Ibadan North Local Government of Oyo state

Markets	Total Bacteria Count (CFU/ml)	Total Count on EMB (CFU/ml)
GATE 1	1.22×10^3	9.70×10^2
GATE 2	1.42×10^3	4.60×10^2
GATE 3	6.70×10^2	1.07×10^2
GATE 4	9.00×10^2	6.20×10^2
BODIJA 1	1.19×10^2	2.70×10^1
BODIJA 2	1.29×10^3	3.30×10^1
BODIJA 3	3.52×10^2	3.20×10^1
BODIJA 4	1.88×10^2	6.50×10^1
SANGO 1	1.74×10^2	2.30×10^1
SANGO 2	6.20×10^1	1.00×10^1
SANGO 3	1.73×10^2	2.40×10^1
SANGO 4	7.60×10^2	3.10×10^1
OJE 1	1.45×10^4	7.60×10^2
OJE 2	3.18×10^3	1.30×10^3
OJE 3	3.72×10^3	6.40×10^2
OJE 4	1.46×10^5	1.18×10^5
MOKOLA 1	2.62×10^4	2.07×10^4
MOKOLA 2	2.26×10^4	1.00×10^4
MOKOLA 3	4.70×10^3	1.62×10^2
MOKOLA 4	1.42×10^4	1.18×10^4

A total of 169 isolates was obtained from twenty raw meat samples collected from retailers in five different markets in Ibadan north local government area of Oyo state and seventy three isolates were identified as *Eschericia coli* based on morphological and biochemical characterizations and with reference toBergey's Manual of Systematic Bacteriology as *E. coli*.

The result ofGelatinase, DNAse and Haemolysis tests *of E.coli* species is shown in Figure 1.The *E. coli* species showed 100% negative reactions to gelatinase and DNAse and 99% negative reaction to haemolysis on blood agar with only isolate B52 showing positive reaction to haemolysis.

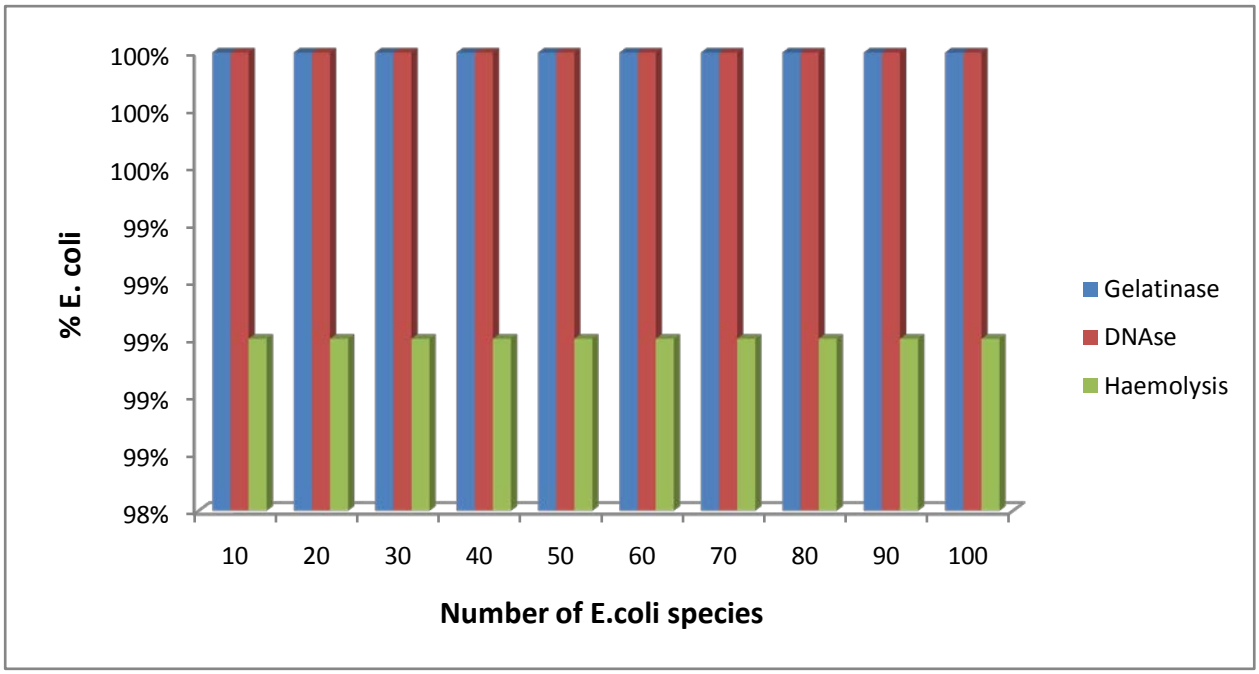

Figure 1: The result of Gelatinase, DNAse and Haemolysis tests *of E.coli* species

The bio-film production by *E.coli*is presented in Figure 2. Eight eighty (88%) of the bacteriaproduced bio-film with 53% showing moderate bio-film production, 26% weak bio-film production and 9% strong bio-film production while 12% were non bio-film producers.

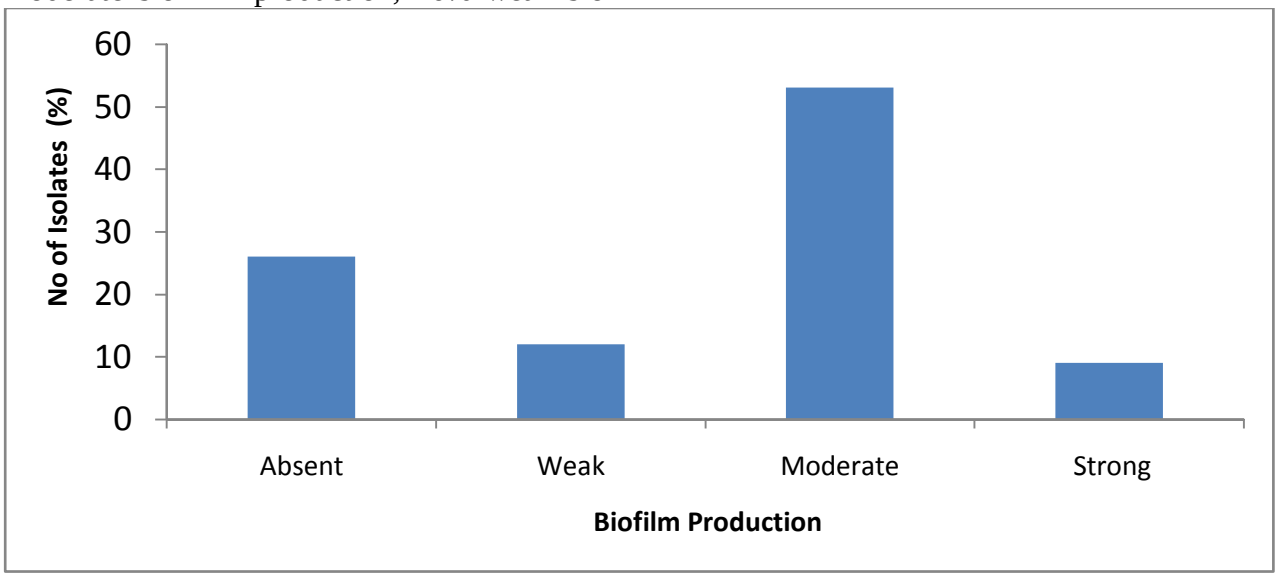

Figure 2: Bio-film Production by *E. coli*

The antibiotic susceptibility pattern of *E.coli*species is shown on Table 2.Eight different antibiotics namely Ceftazidime, Cefuroxime, Gentamicin, Ciprofloxacin, Ofloxacin, Ampicillin, Amoxicillin-clavulanic acid, Nitrofurantoin were used. The result showed that 91.78% of the *E.coli* species was resistant to Ampicillin followed by Gentamicin with 83.56% resistance and Ceftazidime with 64.38% resistance while the antibiotics susceptibility recorded showed that the highest susceptibility of 68.49% was recorded for Nitrofurantoin, while the lowest susceptibility of 1.37% was recorded for Ampicillin.

Table2: Antibiotic susceptibility pattern of isolated*E. coli*

Antibiotics	% occurrence n (%) Susceptible (S)	% occurrence n (%) Intermediate (I)	% occurrence n (%) Resistant (R)
CAZ	7(9.59)	19(26.03)	47(64.38)
CRX	8(10.96)	26(35.62)	39(53.42)
GEN	3(4.11)	9(12.33)	61(83.56)
CPR	16(21.92)	35(47.95)	22(24.13)
OFL	39(53.42)	18(24.68)	16(21.92)
AUG	4(5.48)	29(46.57)	40(47.95)
NIT	50(68.48)	16(21.92)	7(9.59)
AMP	1(1.37)	5(6.85)	67(91.78)

Keys: Ceftazidime (CAZ), Cefuroxime (CRX), Gentamicin (GEN), Ciprofloxacin (CPR), Ofloxacin (OFL), Ampicillin (AMP), Amoxicillin-clavulanic acid (AUG), Nitrofurantoin (NIT).

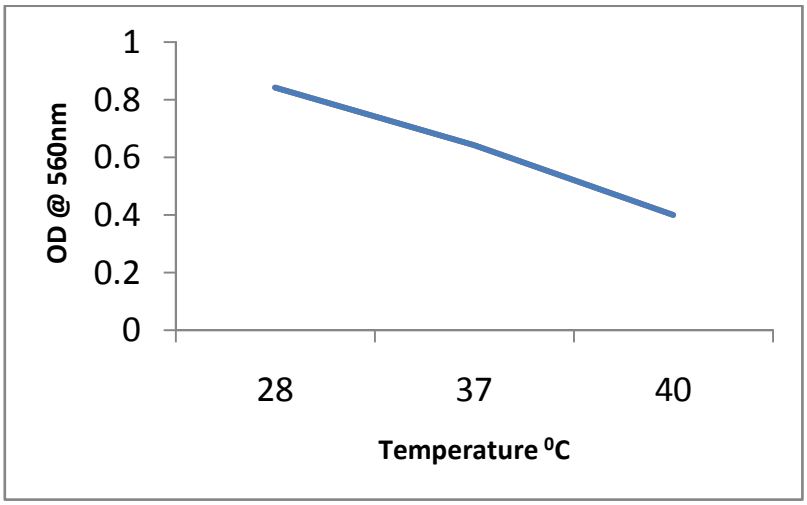

Figure 3: Growth pattern of *E. coli* atdifferent temperatures

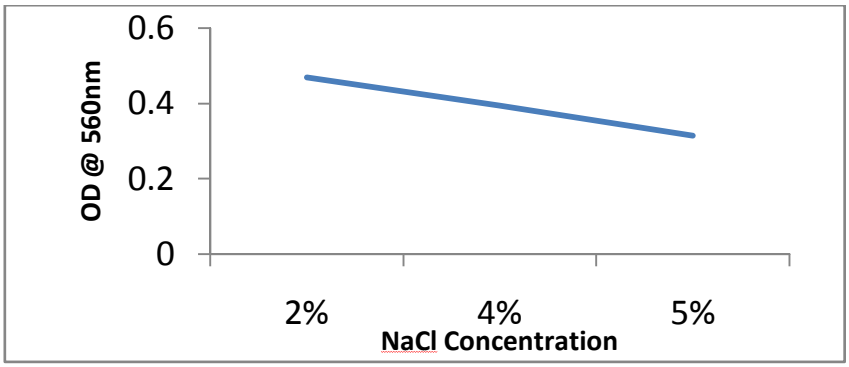

Figure 4.Growth pattern of *E.coli* species at different concentration of sodium chloride

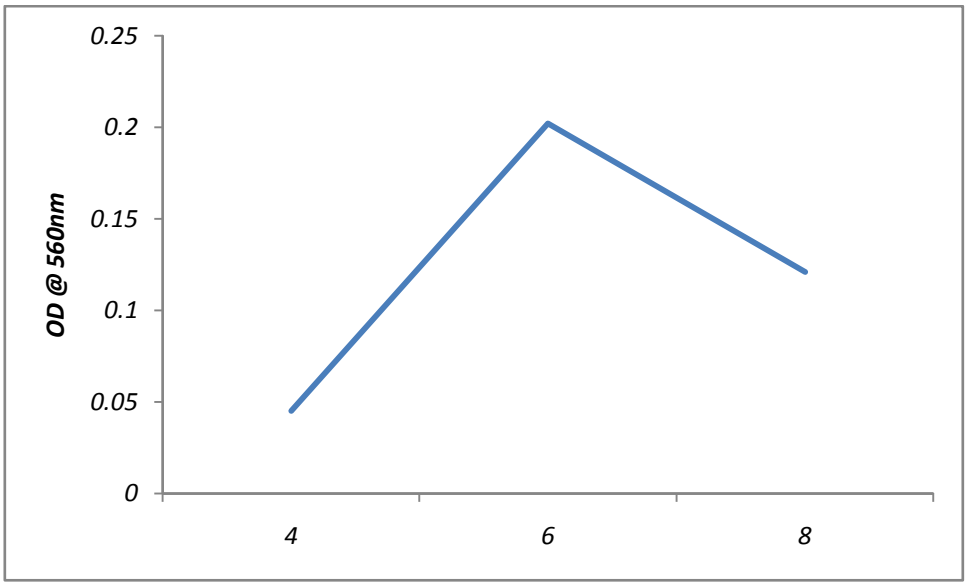

Figure 5: Growth pattern of *E.coli*species at different pH

Figure 3 shows the growth of *E.coli* species at different temperatures.The optimum growth of the *E. coli* specieswas recorded at28^{0}C while moderate growth was also seen within temperature range of 37^{0}C- 40^{0}C.

The sodium chloride tolerance of*E. coli* species is shown in Figure 4.The optimum growth was recorded at 2% NaCl and moderate growth was seen at 4% while the least growth was observed at 5% sodium chloride.

The growth pattern of the *E.coli* species at different pH levels is presented in Figure 5.The optimum growth was recorded at pH 6.0 (OD= 0.202) followed by pH 8.0 (OD= 0.121) and the least was seen in pH 4.0 (OD= 0.045). (OD- Optical Density)

DISCUSSION

Thisstudyprovided empirical information on isolation and phenotypic identification, pathogenicity, antibiotic sensitivity pattern, bio-film formation and physiological behavior of pure cultures of*E.coli* species isolated from raw meat samples collected from selected retailers in Ibadan North local government of Oyo state Nigeria. Out of the One hundred and sixty nine isolates obtained,seventy three were identified based on morphological and biochemical characterization with reference to Bergey's Manual of Systematic Bacteriology as *E. coli*species.Previous reports on the isolation of *E.coli* species from raw meat samples are well documented(Benkerroum*et al*., 2004; Hazarika *et al.*,2004a ;Elimali*et al.*, 2005 ; Bekele *et al.*, 2014).The variation in the number of *E. coli*species isolated from different slaughter houses may be due to difference in hygienic conditions within the slaughter houses (Rahimi*et al.*, 2012; Hazarika *et al.*, 2004b).

However,Thakur *et al.* (2010) reported the isolation of 114 *Campylobacter jejuni* and 78 *Campylobacter coli* from 814 meat samples from different retailers in Iowa , whileSingh and Prakash (2008) isolated 21 *E. coli* species, 12 *S. aureus* species and 13 *L. monocytogenes* from cottage cheese and curd within a period of 1 year from different regions of Agra city. Several researchers such as Direkel*et al.* (2010); Adu-Gyamfi*et al.* (2012); Shekh*et al.* (2013); Erdem*et al.* (2014); have also isolated *E. coli* from minced meat, ground meat, chicken and pork, milk and vegetable salad samples.Reasons such as method of manufacturing, handling and sale which are entirely based on the traditional systems could be adduced for the contamination (Singh and Prakash, 2008). In additionthe unclean hands of workers, poor quality of milk, unhygienic conditions of the manufacturing unit and water supplied for washing the utensils could be sources of acceleration of bacterial contamination of food products apartfrom the

post manufacturing contamination (Elmahmoodand Doughari).

Pathogenicity of bacterial speciesis mainly probably due to the acquisition and expression of various virulence factors especially bio-film formation. However bio-film production by *E.coli* species isolated in this study is relatively lower to that previously reported by Dhanalakshmi*et al*. (2015). In addition,Mah and O' toole, (2001) have shown that 10-1000 times more antibiotics are required to treat an infection caused by a bio-film-associated microorganism. Bio-films provide an ideal niche for the exchange of extra chromosomal DNA responsible for antibiotic resistance, making it a perfect opportunity for emergence of drug resistant pathogens (Donlan and Costerton, 2002). The presence of bio-films in food-processing industry and environments is of economic importance because they confer pathogenicity to these food borne microbes and the ability of the organisms to be easily transmitted from one food product to another (Van Houdt and Michiels, 2010). The findings of Donlan (2002) and Stoodley*et al.* (2002) confirmed the presence of mixed bacterial population or a single bacterial species in a bio-film setting and they contribute significantly to adherence to food surfaces and resistance to removal from the surfaces of substrate by disinfectant. The mechanism of microbial bio-film to resistance of disinfectants can be attributed to slow or partial penetration of the biocide into the bacterial cells, expression of an adaptive stress response by some cells, or differentiation of a small subpopulation of cells into per sister cells (Van Houdt and Michiels,2010).

Hemolysis of sheep RBC is an important criterion for identification of pathogenic strains of *E. coli*. The occurrence of pathogenic *E.coli* in raw meat samples have been reported by Tafida*et al.* (2014) who reported that out of 130 *E. coli* obtained from raw meat samples 4 were pathogenic while Enabulele and Uraih (2009) isolated*E. coli* O157 from raw meat and "suya" in Benin, Nigeria. In addition,Dulo (2014) reported the isolation of 6 (2.55%) E. coli O157:H7 from a total of 235 different samples examined comprising of fecal contents, carcass swabs and environmental samples. Jo *et al.* (2004) reported that human infections caused by *E. coli* O157:H7 have been associated with food products of animal origin. The natural reservoirs of STEC have been confirmed mainly as cattle, sheep, and goats and they are involved in the epidemiology of human infections (Espie*et al.*, 2006; Rey *et al.*, 2006; La Ragione*et al.*, 2008). The existence of *E.coli* O157:H7 and *Salmonella* sp. in raw meat and poultry products constitute a threat to consumers (Sperber, 2005) and available dressing procedures at abattoir cannot be relied upon to get rid of faecal contamination during slaughter.

The antibiotics resistance pattern of *E. coli* to ampicillin in this study is higher than that reported by Abdellah*et al.*(2013); Dulo (2014); Ousman*et al.*(2014); Rasheed *et al.*, (2014) that reported the ampicillin resistance of *E. coli* species isolated from dairy farms, turkey meat, goat meat samples and raw meat respectively. However, Dulo*et al.* (2014) reported higher resistance of *E. coli* to nitrofurantoin and lower resistance to amoxycillin-clavulanic acid than that reported in this study. This observation was similar to the report of Lee (2009) and Schwaiger*et al.* (2012).Buffalo meat, beef and other types of meat are the mostly implicated source of human infection (Capita *et al.*, 2002). The resistance of *E. coli* to antibiotics may be due to its indiscriminate use (Alfredson and Korolic, 2007).

The growth of*E. coli* species at different temperatures observed in this study agrees with the submission of Gill *et al.* (1998), that reported growth of *E. coli*species within a temperature range of 10^0C-40^0C.The growth of *E.coli* species at different pH in this study conforms with the report of Albashan (2009) that confirmed the growth of *E.coli* spp. at pH range of 4.0-8.0. Albashan (2009) reported the growth of *E. coli* O157:H7 and *S. typhi* isolated from apple, ciders and orange juices at pH range of 3.0-9.0.Equally the *E.coli*spp tolerated different sodium chloride concentrations,however,Hrenovic and

Ivankovic (2009) observed that *E. coli* and *Acinetobacter junii* were able to survive and multiply in Sodium chloride (NaCl) for 72 h atconcentrations 5% and 3.5% respectively but could not grow at 10% and 20% NaCl concentrationsrespectively.Physiological studies can provide useful information about parameters that can be effectively used in inhibiting microbial growth during storage.

In conclusion, the results of this study shows that raw meat samples are highly contaminated with food-borne pathogens especially *E. coli*. It is therefore necessary to find effective methods of eliminating orreducing contamination andspread of these food borne pathogens from the production level to the consumer level.This can be achieved through sensitization of the raw meat handlers by healthofficers at the abattoir and the general public about the dangers associated with contamination of meat by microorganisms and sanitary methods that could help reduce their spread. In addition, provision of facilities and equipments needed in the Abattoir such as adequate supply of potable water, hot water, and soap by the relevant authorities. This will help reduce the possibility of contamination.

Regular medical checkup should be done by individuals working in the meat environment to ascertain their health status so as not infect raw meat during processing. In addition, it is necessary that health officers should always monitor and inspect activities in slaughter houses to the level of retailers.

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