Nigerian Veterinary Journal 27 (2):33-40

Biochemical and serological characterization of *Escherichia coli* isolated from colibacillosis and dead-in-shell embryos in poultry in-Zaria-Nigeria.

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SUMMARY

This study was designed to determine the isolation rate, serotypes and biochemical profiles of *E. coli* from colibacillosis and dead-in-shell embryos in Zaria, Northern-Nigeria. The isolation rate of *E. coli* from hatcheries studied were 4.67% and 7.50% from farms of Simtu Agricultural Company and National Animal Production Research Institute (NAPRI) Shika Zaria, Nigeria respectively. Twenty *E. coli* isolates from clinical cases of colibacillosis were also used for this study. The Simtu farm *E. coli* isolates showed 97.5% motility, while isolates from both NAPRI and clinical colibacillosis cases were 100% motile. The results of carbohydrate fermentation were variable without any specific pattern, except for few sugars that had 100% fermentation especially the lactose, ducitol, rhamnose, and xylose of *E. coli* isolates from clinical cases of colibacillosis. The major serotypes recorded from clinical cases of colibaccillosis were O8:K50 and O9:K30. Serotypes obtained from the dead-in-shell embryos were O78:K80, O8:K50, O9:K30, and O26:K60. Untypable isolates made up the greater percentage of *E. coli* strains studied. The antibiotic susceptibility testing showed that most of the isolates were resistant to more than one antibiotic. Majority of the isolates were sensitive to ciprofloxacin (85%) of the clinical cases and 100% of each of the Simtu and the NA.PRI. farms. In conclusion, this study has revealed the involvement of several *E. coli* serotypes in colibaccillosis and dead-in-shell embryos. It is recommended that measures aimed at reducing the emergence of resistant strains of *E. coli* be instituted in all the farms.

KEY WORDS: Escherichia coli, Serotypes, biochemical profiles, and dead-in-shell embryos.

INTRODUCTION

Colibacillosis is one of the principal causes of mortality and morbidity in chickens and turkeys resulting in significant economic losses to poultry industry. *Escherichia coli* cause different types of disease syndromes in poultry, including: acute colisepticaemia, sub-acute fibrinopurulent synositis, yolk sac infection, cellulitis, swollen head syndrome and coligranuloma (Gomis *et al.*, 2001; Allan *et al.*, 1993).The most common form of colibacillosis is characterized by an initial respiratory infection (air sacculitis) in 3 to 12-week-old broiler chickens and turkeys, which is frequently followed by generalised septicaemia, perihepatitis, and pericariditis (Bopp *et al.*, 2005). Infection is generally enhanced or initiated by predisposing factors, such as mycoplasmas or viral infections (Gomis *et al.*, 2001; Bopp *et al.*, 2005).

Avian enteropathogenic *E. coli* (AEPE) for poultry commonly belong to certain serogroups particularly serogroups O78, O1, and O2, and to some extent O15 and O55 (Gross, 1994; Chart *et al.*, 2000). Virulence attributes such as: the aerobactin iron sequestration (Dozois *et al.*, 1992), capsules (e.g. K1), lipopolysaccharide, cytotoxins such as -haemolysin(Ngeleka *et al.*, 1996) resistance to the bactericidal effect of serum(Dho-Moulin *et al.*, 1990) and fimbrial (pili) adhesion(Arne *et al.*, 2000; Jeffrey *et al.*, 2002) are associated with pathogenic *E. coli* that cause colisepticaemia. Reduced hatchability is one of the major problems in the hatchery industry, which has adversely affected the rapidly expanding poultry production in Nigeria. This has been related to nutritional deficiencies and infertility in breeders, faulty incubation, embryonic malpositions and bacterial infections of embryo (Wooley *et al.*, 2000; Akinyemi and Ojeh, 1982; Falade, 1977). Bacterial infection of the embryo is a major cause of reduced hatchability, early chick mortality and poor performance (Wooley *et al.*, 2000; Kabilika and Sharma, 1997). In Nigeria, Akinyemi and Ojeh (1982) and Falade (1977) isolated some bacteria species including *E. coli* from infected chicken embryo in Ibadan, Oyo State. Although numerous studies have been conducted on the isolation of *E. coli* in poultry in Nigeria, there is paucity of information on biochemical profiles, serogroups and antibiotic susceptibility of this important agent of mortality and morbidity in poultry. The present study was undertaken to investigate biochemical and serological properties of *E. coli* isolated from cases of colibacillosis, and dead-in-shell embryos in Zaria, Nigeria and their susceptibility to antimicrobial agents.

MATERIALS AND METHODS

Sample collection and isolation of *E. coli* from dead-in-shell embryo.

A total of 600 unhatched and unpipped eggs were selected from five batches of hatch from a private hatchery, Simtu Agricultural Industrial Limited, Zaria and a government owned hatchery unit in National Animal Research Institute (NAPRI) of Ahmadu Bello University, Zaria, Kaduna State, Nigeria. Selected eggs for hatching were candled on the 6th day of incubation to eliminate infertile eggs. The eggs were again candled on the 18th day of incubation. The embryonated eggs that had died between the 6th and 21st day of incubation were used for this study. All samples were macroscopically examined. Eggs with cracks and those embryos that pipped the shell but failed to emerge were discarded to minimize the incidence of external contamination. The surface of each egg was disinfected by cleaning with disinfectant, chlorohexidine (Salvon^{R}) solution and dried with ethyl alcohol for 15 minutes. Flamed wire loop was used to take about 0.2ml of the yolk contents and streaked on MacConkey agar for primary isolation. This was then incubated at 37^{o}C for 24h under aerobic conditions.

Sample collection and isolation of *E. coli* from clinical cases of colibacillosis

Birds that died from suspected cases of colibacillosis and those sacrificed for confirmatory diagnosis from flock outbreak from clients that submitted birds to the Avian Unit of the Veterinary Teaching Hospital, ABU, Zaria were used for this study. The tissues and organs with lesions were seared with hot spatula and sterile cotton swab was used for

sampling collection for culturing (liver, gallbladder, spleen, air sac, and pericardium) from clinical cases of suspected colibacillosis.

Identification of *Escherichia coli*

Bacteria were identified on the basis of their cultural characteristics, morphological and physiological properties. For example on MacConkey agar, colonies appeared as button-like, pinkish colouration (lactose fermenter) and on Eosin methylene blue agar the colonies appeared as greenish metallic sheen. Following identification the colonies, were sub-cultured on nutrient agar slants for storage at 4°C for further studies. The colonies were then subjected to biochemical tests as described by Bopp *et al* (2005).

Biochemical characterization

E. coli isolates were subjected to standard biochemical tests, including catalase, indole, motility, hydrogen sulphide production, carbohydrates fermentation, phenylalanine deaminase, bile esculin hydrolysis, methyl red, Voges Proskauer, citrate, urease and gelatine liquefaction as previously described in detail by Gomis *et al* (2001).

Fermentation of carbohydrates By *E. coli* isolates

The *E. coli* isolates were characterized their ability to utilize the following sugars: maltose, lactose, sucrose, dulcitol, adonitol, salicin, raffinose, dextrin, xylose, rhamnose and mannitol. The indicator used for sugar fermentation (Bromothymol blue broth).The broth base was prepared by dissolving peptone (10g), sodium chloride (5g) and bromothymol blue (0.018g) in 1 litre of distilled water. Each sugar solution was prepared by dissolving 1% of the corresponding sugar in the broth base medium, only salicin was prepared at 0.5%. Each *E. coli* isolate was inoculated into prepared sugar medium and incubated at 37°C for 24h. The test was recorded as positive when the medium turned from bluish colour to yellow, while for negative reaction the medium remained blue (Gross, 1994; Bopp *et al.*, 2005).

Serotyping

Eighty-five of these strains were serologically typed in detail in South Africa. Serotyping of the isolates was done using standard slide agglutination tests with antisera against somatic antigen groups according to standard methods described by Orskov *et al* (1977).

Antimicrobial Susceptibility tests

Fifty-two *E. coli* isolates made of twelve from clinical cases and twenty from dead-in-shell embryos from each farm were subjected to *in vitro* antimicrobial susceptibility testing. The *E. coli* isolates were tested against 10 and 9 antimicrobial agents for clinical cases of colibacillosis and dead-in-shell embryos respectively. The selection of antibiotic disk concentrations and interpretation of the zone size were done as recommended by the manufacturers (Oxoid, UK) and National Committee for Clinical Laboratory Standards (NCCLS, 1990). The following antibiotic disks were used: ciprofloxacin (5 g), sulphamethazole-trimethroprim (25 g), streptomycin (10 g), penicillin G (10 unit), tetracycline (30 g), chloramphenicol (30g), ceftrixazone (30 g), cephalothin (30 g), ampicillin (10 g), amoxicillin (25 g).

RESULTS

In this study, *E. coli* was isolated from 28 (4.7%) And 45 (7.5%) dead-in-shell embryos from Simtu and NARPI hatchery farms, respectively. Twenty *E. coli* isolates were randomly selected from clinical cases of colibaccillosis from positive culture of poultry samples submitted to Microbiology laboratory of Department of Veterinary Pathology and Microbiology, Zaria, Nigeria. The Simtu farm *E. coli* isolates showed 96.43% motility, while 100%of the isolates from both NAPRI and clinical colibacillosis were motile. The results of the bile aesculin hydrolysis showed that 46.4% of the Simtu isolates, 24.4% of the NAPRI and 25% of the clinical colibacillosis hydrolysed bile aesculin, respectively.

All the isolates examined fermented lactose on

MacConkey and showed greenish metallic sheen on Eosin methylene blue agar. The results of carbohydrate fermentation was variable without any specific pattern, except for a few sugars that had 100% fermentation, especially the lactose, ducitol, rhamnose, and xylose for *E. coli* isolated from clinical cases of colibacillosis (Table I). The *E. coli* isolates from dead-in-shell embryos from Simtu farm showed 100% fermentation for xylose, ducitol and lactose. The NAPRI isolates showed 100% fermentation rate for lactose and 97.8% fermentation rate for each of the following sugars (xylose, rhamnose and ducitol (Table II).

All (100%) isolates of *E. coli* from dead-in-shell embryos were sensitive to ciprofloxacin while 17(85%) of isolates from clinical cases were sensitive to this drug. None of the isolates was sensitive to penicillin and cephalothin (Table III). The 93 *E. coli* isolates obtained from clinical cases of colibacillosis and dead-in-shell embryos were serotyped. Twenty-two of the 93 isolates were assigned to O serogroups. Five isolates were not analysed, while the remaining 66 isolates analysed were found to be non-typeable rough strains. The 22 typeable isolates were distributed among the O serogroups as follows O8:K50 (5),

TABLE II. Carbohydrates fermentation of various *E. coli* isolates

Carbohydrates	Clinical Cases of Colibacillosis (n=20)	Dead-in- shell Embryos from Simtu farm(n=28)	Dead-in- shell Embryos from N.A.P.R.I. farm(n=45)
Xylose	100%	100%	97.8%
Mannitol	95%	92.5%	93.3%
Raffinose	95%	85.7%	80%
Sorbitol	75%	75%	91.1%
Adonitol	10%	32.1%	17.8%
Rhamnose	100%	96.43%	97.8%
Lactose	100%	100%	100%
Sucrose	65%	75%	82.2%
Maltose	95%	92.9%	68.9%
Dextrin	85%	75%	46.7%
Ducitol	100%	100%	97.8%
Salicin	65%	60.7%	77.8%

TABLE III. *In vitro* Antibiotic susceptibility of *Escherichia coli* isolated from dead-in-shell embryos and colibacillosis.

Antibiotic Disc potency (µg)	Resistance		
	Clinical Colibacillosis Cases(n=12)	Simtu Farm Isolates(n=20)	N.A.P.R.I Isolates(n=20)
Tetracycline 30µg	60%	19%	81%
Streptomycin 10µg	90%	75%	81%
Chloramphenicol 30µg	70%	NT	NT
Ampicillin 10µg	80%	88%	31%
Cephalothin 30µg	100%	100%	100%
Penicillin 10unit	100%	100%	100%
Amoxicillin 25µg	65%	94%	38%
Ciprofloxacin 5µg	5%	0%	0%
Ceftriazone 30µg	75%	63%	25%
Sulfamethoxaazole-Trimethroprin25µg	70%	50%	75%

NT=Not tested

TABLE IV. Various Serotypes of *E. coli* isolates

Serotypes	Clinical Cases of colibacillosis	Dead-in-shell Embryos Simtu Farm	Dead-in-shell Embryos N.A.P.R.I farm	Total
O8:K50	2	2	1	5
O9:K34	-	-	3	3
O9:K9	-	-	2	2
O9:K28	-	-	1	1
O99:K	-	-	1	1
O8:K	-	-	1	1
O4:K3	-	1	-	1
O26:K60	-	-	1	1
O112:K68	-	-	1	1
O137:K79	-	-	1	1
O13:K11	-	1	-	1
O78:K80	-	1	-	1
O8:K41				
Rough	14	20	32	66
Not included for typing	1	3	1	5
Total	20	28	45	93

DISCUSSION

The isolation rates of *Escherichia coli* from dead-in-shell embryos from Simtu farm and NAPRI were 4.7% and 7.5%, respectively. The findings are similar to those of Kabilika and Sharma, (1997), Grosheva (1971), Orajaka and Mohan (1986). The variation in the percentage of *E. coli* isolates from (4.7%) Simtu farm and (7.5%) from NAPRI may be partly related to the prophylactic and therapeutic use of antibiotics, vaccination for respiratory viruses, and improved hatcheries sanitation. The biochemical profile of *E. coli* isolated from cases of colibacillosis and dead-in-shell embryos was similar to those previously reported for colibaccillosis and dead-in-shell embryos (Gomis *et al.,* 2001; Bopp *et al.,* 2005).

The finding obtained in this study disagreed with the report of Cloud *et al.,* (1985) and Orajaka and Mohan (1986) who recorded high incidence of serotypes O1, O2 and O78 in cases of colibacillosis and dead-in-shell embryos. In this study, serotypes O8, and then O9 and O78 in that order were most frequently isolated. Falade (1977), in Oyo State, Nigeria, serotyped *E. coli* isolates and serogroups O141 and O139, which are not among the known serogroups normally associated with pathogenic lesions in poultry in his study. None of these serogroups were isolated in the present investigation.

Serogroup O86 recorded in the present study has previously been reported to be highly pathogenic for 3-5 day-old chicks (Burkhanova, 1980). The O86 and O26 groups are among the enteropathogenic *E. coli* known to be associated with infant haemorrhagic colitis and bloody diarrhoea (Cravioto *et al.,* 1979). This is suggestive of the possible zoonotic effect of some of the *E. coli* serogroups associated with dead-in-shell embryos. The O8 serogroup has also been associated with hatchery losses and early chick mortality in India (Venugopalan *et al.,*

1974; Arunachalan *et al.,* 1974). Also Hinton and Linton (1982) had reported the association of colibacillosis to the presence of O8 and O9 in South Africa.

There were rough untypeable strains of *E. coli* (66 of the 88 isolates analysed) in the present study. This finding is in agreement with Cloud *et al* (1985) who reported 63.5% untypeable strains of *E. coli* from yolksac disease. However, Orajaka and Mohan (1986) found only 26% untypeable *E. coli* strains from dead-in-shell embryos. Very little information is available on the association of rough untypeable *E. coli* strains with embryonic mortality. However, Rosenberger *et al* (1985) reported that O2 serotypes and untypeable *E. coli* of avian origin are among virulent avian *E. coli* in colibacciollosis. This observation was not confirmed in the present study.

The results obtained in this study on antibiotic susceptibility suggest that multiple antibiotic resistances are widely spread among the local strains of *E. coli* from poultry. These observations agreed with the reports by Blanco *et al.,* (1997) and Cloud *et al.,* (1985): the development of antibiotic resistance among bacterial agents has been attributed to irrational use of these drugs in veterinary practices. It is also very significant to note that almost all the *E. coli* isolates showed very high resistance to streptomycin, tetracycline and ampicillin. This is of serious concern because these drugs are still considered the most recommended for the treatment of colibacillosis in both animal and man. There is, therefore, an urgent need to reverse this notion in the light of present study with regards to the sensitivity pattern of each particular isolates of *E. coli.*

In the present study, most isolates were highly sensitive to ciprofloxacin and ceftriazone and this may be due to the fact that these drugs are not used in poultry industry in Nigeria. However, many of the other antibiotics that are used extensively in poultry industry were less effective (Cloud *et al.,* 1985; Ojeniyi, 1989). It will be proper to change the drug prescription to these fluorated piperazinyl - substituted quinoline derivates in the light of the information obtained in the present study. Ciprofloxacin and other fluorated piperazinyl-substituted quinoline have been used by the poultry farmer only recently in Zaria. Many of the other antibiotics, such as the sulpha compounds, tetracycline chloramphenicol, penicillin and cephalothin that have been used extensively in the poultry industry were less effective. Although ceftrixazone is rarely used, the high incidence of resistance to this compound can be associated with a transferable plasmid also carrying resistance to the tetracyclines (Ojeniyi, 1989).

It is also disturbing to note that chloramphenicol which is the drug of choice for the treatment of colibacillosis and other enteric pathogens in animals showed high resistance against *E. coli* from clinical cases of colibacillosis in this environment. Generally, majority of the isolates were highly resistant to the common, less costly antibiotics used in poultry industry. Certainly other methods for controlling *E. coli* should be evaluated, so as to minimise the emergence of resistant strains in order to reduce the cost of prophylactic and therapeutic treatment programs.

CONCLUSION

In conclusions this study document colibacillosis and dead-in-shell embryo, in northern, Nigeria. There was no difference observed in the *E. coli* serotypes isolated from cases of colibacillosis and dead-in-shell embryos. Serotype O8:K50 was most frequently encountered in the study followed by O9:K34 then by O9:K9 among others. The majority of *E. coli* strains were roughed so they could not be typed because the smooth strains could be typed easily. The ecology of the *E. coli* isolates will be important in the farms studied. The serological, biochemical and antibiotics sensitivity characterization of *E. coli* isolates associated with recently diagnosed avian colibacillosis and dead-in-shell embryos should be used in developing new methods of control of these diseases

ACKNOWLEDGEMENTS

We thank Mr. Bitrus, Mr. Dodo, Mr. Michael and Miss Hellen for their assistance during the processing of all *E. coli* isolates sent to Bacteriology Laboratory, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria. We Also appreciate the authorities of the Department of Bacteriology, Onderstepoort Veterinary Institute, South Africa.

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