PATHOLOGICAL STUDY ON COLIBACILLOSIS IN CHICKENS AND DETECTION OF *ESCHERICHIA COLI* BY PCR

N.S. Tonu, M. A. Sufian, S. Sarker, M.M. Kamal, M.H. Rahman and M.M. Hossain*

Department of Pathology, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh

ABSTRACT

The aim of the present study was to detect the pathogenic *Escherichia coli* (*E. coli*) through pathological study of the colibacillosis affected birds. These isolated *E. coli* were further confirmed by PCR using specific primer. For this purpose, a total of 20 swabs (10 from lung and 10 from intestine of 10 dead birds) were collected in sterile nutrient broth. The histopathological samples were collected in 10% buffered neutral formalin. The used methods were histopathology, isolation and identification of *E. coli* by conventional methods and as well as by PCR method. A total of 10 isolates of *E. coli* from 20 swabs of lung and intestine was characterized by conventional routine methods of bacteriology. Gross pathological lesions of all lungs in the present investigation were congested and consolidated. Duodenum showed congestion and hemorrhages with excess mucus in the luminal surface of it. Microscopically, all the lungs showed severe congestion, infiltration of heterophils, macrophages and lymphocytes in the wall of bronchus as well as in the peribronchial alveoli. *E. coli* infected all the duodenum showed severe infiltration of leukocytes mainly heterophils, lymphocytes and macrophages in the submucosa of the duodenal wall. In this study, DNA of 8 isolates out of 10 isolated *E. coli* organisms was amplified by PCR using ECO-f and ECO-r primer targeting 16S ribosomal DNA and found 585 bp amplicon which is specific for *E. coli* with enteroinvasive type confirmed by histopathological lesions in duodenum. Further investigation should be focused on serotyping and detection of genes of *E. coli* which are responsible for pathogenicity of the organism.

Key words: Colibacillosis, histopathologcal, chickens, PCR

INTRODUCTION

Bangladesh livestock population statistics indicates poultry as the most important species of birds in this country. A total of 98.15% of poultry are kept in rural area and they are scavengers (BBS, 1987). For the last several years, poultry rearing has been developed as an industry in Bangladesh. With great expansion of the poultry rearing and farming, colibacillosis has become wide spread problem in Bangladesh like other areas of the world (Talha *et al.,* 2001; Islam *et al.,* 2003 and Rahman *et al.,* 2004). Heavy economic loss occurs in both broiler and layer due to morbidity, mortality, reduced production and chick quality. Mortality may rich up to 94% in severe outbreak of colibacillosis (McPeake *et al.,* 2005 and Biswas *et al.,* 2006).

The organisms of *E. coli* are divided into pathogenic and nonpathogenic based on their ability to cause diseases. Pathogenically, *E. coli* strains are due to the presence of one or more virulence factors including invasiveness factors invasins, heat labile, heat stable enterotoxins, verotoxins and colonization factors or adhesins (Smith and Haibs, 1967). Pathogenic *E. coli* are divided into two types namely as enteropathogenic *E. coli* and uropathogenic *E. coli.* Further pathogenic *E. coli* are grouped into enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAggEC), enterohaemorrhagic *E. coli* (EHEC). Uropathogenic *E. coli* cause 90% of the urinary tract infection. The bacteria colonize from the faeces or perineal region and ascend the urinary tract to the bladder of the animals (Carter, 1986).It is quite difficult to differentiate the pathogenic strains from that of the nonpathogens on the basis of antigenic, structural and biochemical characteristics (Dupont *et al.,* 1971). It is not known whether the pathogenic activity is determined entirely or in part by the host. If the host plays the dominant part, then any normal or wild strain of *E. coli* will be able to assume a pathogenic role. Therefore, it urges for the separation of pathogenic strains from the nonpathogenic one (Levine, 1984).

Pathogenic *E. coli* are usually identified by detection of a specific virulence factor or of a serotype associated with a virulence factor. Over 700 antigenic types (serotypes) are recognized based on 0, H and K antigens. Different serological test such as fluorescent antibody test (FAT), enzyme-linked immunosorbent assay (ELISA) test, serum agglutination test (SAT), growth inhibition test (GIT) are used for the detection of enterotoxigenic and enteropathogenic *E. coli* (Klipstein, 1984). Different molecular techniques such as nucleic acid hybridization and polymerase chain reaction (PCR) studies can also be used to detect pathogenic *E. coli* and DNA probes directed at a number of genes have also been developed (Wani *et al.,* 2003). In vitro pathogenicity test for *E. coli* is determined by using congo red binding activity (Ahmad *et al.,* 2009).Various parameters including the prevalence, isolation identification and epidemiological investigation of all type of pathogenic and nonpathogenic *E. coli* were studied in Bangladesh by different authors (Choudhury and Rahman, 1967; Mostofa, 1998). Detection of avian pathogenic of *E. coli* was not systematically and properly focused by the previous research works in the context of Bangladesh. The use of molecular techniques has several advantages compared with the classical culture methods for enumerating bacteria; one major advantage is the rapidity and sensitivity of the determination compared with culture methods. Perhaps the most serious drawback of the culture- based methods is that only a small fraction of bacteria can be found; up to 99% of the bacteria in many environments fail to grow under artificial conditions (Amann *et al.,* 1995). Amplification of DNA sequences unique to organisms using the PCR improves both the speed of detection and the level of sensitivity at which organisms can be detected and has been increasingly used to identify several bacterial species from food and clinical samples. Another advantage is that PCR is not dependent on the utilization of a substrate or the expression of antigens, thereby circumventing phenotypic variations in biochemical patterns and lack of detectable antigens. The use of PCR for rapid diagnosis is inevitable for the control of disease in poultry farms of Bangladesh.

To our knowledge many investigations have been performed on pathogenicity test of *E. coli* through animal inoculation but molecular detection of avian pathogenic *E. coli* has not been performed in our country. In this study a primer set targeting *E. coli* 16S rDNA gene has been used for the detection of *E. coli.* Therefore, the present research work was performed with these objectives (a) pathological studies of colibacillosis affected dead birds. (b) isolation and identification of *E. coli* from the collected sample of dead birds. (c) detection of pathogenic *E. coli* using specific primer.

MATERIALS AND METHODS

Samples
A total of 10 dead birds from Krishibid Poultry Farm, Bhaluka (8 birds) and S.K. Diagnostic Center (SKDC), Mymensingh (2 birds) and a total of 20 swabs samples (10 from lung and 10 from intestine) were collected from necropsy cases during the study period from June 2009 to January 2010.

Culture in different media
All lung and intestinal swabs were placed in nutrient agar plate and incubated for overnight at 37°C for the growth of the organisms. After primary culture of the organisms, a small amount of inoculum from nutrient agar were subcultured in the nutrient agar and MacConkey agar to observe the colony morphology. Characteristic colony morphology of the organisms indicating the features of *E. coli* was selected for subculture on EMB agar (Carter, 1986). Morphological characteristics (shape, size, surface texture, edge and elevation, color, opacity etc. of the suspected colonies on different agar media developed within 18 to 24 hours of incubation were carefully studied and recorded.

Gram's stain
Gram's staining was performed as per recommendation of Merchant and Packer (1967) to determine the size, shape and arrangement of bacteria.

Motility test
The motility test was performed according to the method described by Cowan, (1985) to differentiate the motile bacteria from the non-motile one by hanging drop preparation.

Sugar fermentation test

The sugar fermentation test was performed by inoculating 5 ml of nutrient broth culture of the organisms into the tubes containing different sugar media and incubated for 72 hours at 37°C. Acid production was indicated by the color change from red to yellow of the medium and the gas production was noted by the appearance of gas bubbles in the inverted Durham's tube.

Methyl red test procedure

After incubation 2-4 drops of methyl red solution were added to the test which was incubated for 5 days at 37°C for MR test. Positive test was indicated by the persistence of red color, indication of acidity and the negative one by the yellow color.

Voges-Proskauer (V-P) test procedure

The VP test was performed by adding 0.6 ml of VP reagent-1 and 0.2 ml of VP reagent-2 for each ml of culture. The ingredients were mixed thoroughly and allowed to stand for a while. The appearance of pink color indicated to positive test.

Gross lesions

The postmortem examination was performed for the lung and intestine of dead birds. At necropsy, gross tissue changes were observed and recorded carefully, and representative tissue samples containing lesions were fixed in 10% neutral buffered formalin for histopathological studies (Stubbs, 1954).

Histopathology

The formalin-fixed tissues were trimmed, processed, sectioned and stained as per standard procedure (Luna, 1968). Specific lesions containing samples were used in histopathological study.

Detection of *Escherichia coli* (*E. coli*) by PCR
Oligonucleotide primers

For PCR analysis of locally isolated *E. coli*, oligonucleotide primers targeting 16S rDNA gene (Candrian *et al.,* 1991; Amit-Romach *et al.*, 2004) (Science Park Rd. #01-23, The Gemini, S'pore) ECO-f and ECO-r were used (Table 1).

Table 1. Primers were used for the detection of the *E. coli*

Primer	Primer sequence (5′-3′)	Amplification products (bp)
ECO-f	GACCTCGGTTTAGTTCACAGA	
ECO-r	CACACGCTGACGCTGACCA	585

DNA extraction and Quantification of DNA concentration

DNA was extracted from *E. coli* isolate using Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) and extracted DNA was quantified by using spectrophotometer (DU-640, Beckman, Germany) on the basis of optical density ratio at 260: 280 nm.

DNA amplification

PCR amplification was performed in a final volume of 20µl containing 4µl (50ng/µl) of DNA template, Taq buffer- B 2µl, dNTPs 2µl (250mM), primer-F 1µl (100mM), primer-R 1µl (100mM), Taq DNA polymerase 0.4µl (3U/µl) and 8.4µl nuclease free water (Science Park Rd.#01-23 The Gemini, S'pore). Three independent reactions, with the primers, were made for DNA template. Amplification was carried out in Gene amplification PCR system 9600 Thermocycler (Master cycler eppendorf, Germany). Initial denaturation was at 94^0C for 3 min., followed by 35 cycles for 94^0C for 30 sec., annealing at 60^0C for 1 min and extension at 68^0C for 2 min, with a final extension at 68^0C for 7 minutes and hold for 4^0C.

Agarose gel Electrophoresis

Amplified products were separated by electrophoreses on 1% agarose gel containing ethidium bromide. The agarose gel was prepared using 1gm agarose powder (Fisher Biotech, New Jersey, USA and Genei, Banglore India), 100ml 1X TAE buffer and 5µl ethidium bromide. Agarose gel electrophoresis was conducted in 1X TAE buffer at 100 V for 35 minutes. 100 bp ladder (Ggibco BRL) as molecular weight marker and loading dye (0.25% xylene cyalon, 0.25% bromophenol blue, 30% glycerol and 1 mM EDTA) were used for electrophoresis. Bands were observed under UV light on a transilluminator and photographed by image documentation system (Labortechnic, Germany).

Photomicrography

Photomicrography was taken at the Department of Pathology using photomicrographic camera (Olympus PM-C 35 Model) onto fitted with Olympus microscope (Olympus, Japan).

Data interpretation

At the end of the study period all findings were compiled, scrutinized and analyzed for comprehensive interpretation.

RESULTS AND DISCUSSION

The colony characters of identified bacteria

The present study was conducted primarily identification of pathogenic *E. coli* by molecular detection in dead chickens. *E.coli* is an adaptive species that is both a commensal resident of the intestine and a versatile pathogen of humans and other animals, causing enteric infections and particular pathologies in different animal species (Dozois *et al.*, 2003). The stress factors of any types may enhance the virulence of *E. coli* with production of diseases (Talha *et al*., 2001). Therefore, for animal and human health, there is great importance in developing a method that will enable accurate and rapid identification of the above bacteria. A total of 10 isolates of *E. coli* from 20 swabs of lung and intestine was characterized by conventional routine methods of bacteriology. Rose pink colored colonies on MacConkey agar (Fig. 1) and greenish colonies with metallic sheen on EMB agar produced by the organisms (Fig.2) after overnight incubation were confirmed as *E. coli.* In this study, the colony characters of all isolated *E. coli*, were the production of metallic sheen on the EMB agar and rose pink color colonies on the MacConkey agar which corresponded with the findings of other (Sharada *et al.,* 1999).

Gram's stain

Light microscopic examination of all 10 isolates after Gram's staining revealed Gram-negative, pink colored, short rod shaped organisms arranged as single or in pair (Fig.3). The morphology of the isolated bacteria from 10 isolates exhibited Gram negative, pink color, short rod to coccoid shape and in the hanging drop technique all the isolates revealed motile. These findings supported the findings of several authors (Merchant and Packer, 1967 and Khoskhoo and Peighambari, 2004).

Motility test

All 10 isolates of *E. coli* isolates were found to be motile with 'hanging drop' preparation under microscope. These findings supported the findings of several authors (Merchant and Packer, 1967 and Khoskhoo and Peighambari, 2004).

Sugar fermentation test and Other biochemical tests

All 10 isolates of *E. coli* fermented dextrose, lactose, maltose and mannitol with the production of acid and gas (Table 2) but did not ferment inositol . Acid production was indicated by the color changes of the sugar media from reddish to yellow and the gas production was noted by the accumulation of gas bubbles in the inverted Durham's tube (Table 2). All the isolates were methyl red positive and Voges-Proskauer test negative (Table 2). In the present study, the entire isolated *E. coli* organisms revealed a complete fermentation of those sugars and the organisms were motile but did not ferment inositol. These findings also supported the findings of several authors (Shinebaum and Cooke, 1985 and Beutin *et al*., 1997). In the present study, the results of methyl red test of 10 isolates of *E. coli* were positive but Voges-Proskauer test was negative which were also similar to the findings of other author (Thomas, 1988).

Table 2. Biochemical characteristics of *E. coli*

No. of isolates	Methyl red test (MR test)	Voges-proskauer test (VP test)	Motility test	Sugar test				Name of isolated bacteria
				DX	L	ML	MN	
10	+	–	+	AG	AG	AG	AG	*E. coli*

Legends: DX= Dextrose, L = Lactose,   ML = Maltose,   MN = Mannitol,   AG = Acid and gas,   + = Positive, – = Negative

Gross lesions

 Postmortem findings revealed congested and consolidated lung (Fig. 5) in some chickens. The duodenum showed mucus (Fig.4), congestion and hemorrhages (enteritis) and all these lesions were categorized into septicaemic from. The severity of gross lesions was variable in all the dead birds in the present study corresponded with the findings of other investigators (Talha *et al*., 2001; Islam *et a*l., 2003 and Ghosh *et al*., 2006).

Microscopic lesions

 The section of lungs showed severe congestion, infiltration of heterophils, macrophages and lymphocytes in the wall of the bronchus as well as in the peribronchial alveoli (Fig.6). In lungs granuloma were also present. In duodenum, severe infiltration of leukocytes mainly heterophils, lymphocytes and macrophages in the submucosa of the duodenal wall were observed. Enlarged mucosal glands were also observed (Fig. 7). Microscopically, all dead birds did not reveal homogenous severity of lesions in all organs in the present study corresponded with the findings of others (Talha *et al*., 2001; Islam *et a*l., 2003 and Ghosh *et al*., 2006).

 E. coli causes severe types of disease conditions such as colisepticemia, coligranuloma (Hjarre's disease), avian cellulites (inflammatory process), air sacculitis, swollen head syndrome, enteritis, pericarditis, peritonitis, salpingitis, osteomyelitis/ synovitis, panophthalmitis and omphalitis / yolk sac infection (Chauhan, 1996 and Barens and Gross, 1997). In the present study all the reported forms of colibacillosis were not recorded. However, the recorded forms of colibacillosis could be categorized into septicaemic form with enteritis and air sacculitis. *E. coli* singly does not produce typical gross lesions. The lesions are most prominent when simultaneously infected with other organisms such as *Mycoplasma*. *E. coli* can produce diseases becoming attached with mucosal epithelia and another form by invasion to the mucosal epithelia. The recorded lesions of intestine were in the form of degeneration, necrosis and desquamation of mucosal epithelia associated with severe inflammation. On the basis of the recorded lesions, the form of colibacillosis in the present study could be categorized into enteroinvasive form of colibacillosis. These types of histopathological lesions in recorded forms of colibacillosis were supported by different authors (Talha *et al*., 2001; Islam *et a*l., 2003 and Ghosh *et al*., 2006).

Detection of *Escherichia coli* (*E. coli)* by PCR

 Locally isolated 8 isolates (out of 10) of *E. coli* were tested by ECO-f and ECO-r primer and showed 585-bp products from each field samples after 1% agarose gel electrophoresis (Fig. 8). Traditionally, diagnosis of colibacillosis was carried out by isolation and identification of *E. coli*. The isolation and identification depend upon the culture of the organism using different selective media and biochemical tests. Instead of biochemical and ELISA tests, PCR and its related method have been reported to identify *E. coli.* The PCR is used as a highly sensitive and specific test for the presence of pathogenic bacteria in clinical specimens (Cohen *et al.,* 1993). PCR is also more rapid, reliable than traditionally culture methods (Carli *et al*., 2001).We also found the similar results in the present study. In this study, the isolated *E. coli* organisms from all collected birds were grown in nutrient broth, extracted DNA and amplified by PCR using ECO-f and ECO-r primer targeting *E. coli* 16S ribosomal DNA and found 585 bp amplicon after 1% agarose gel electrophoresis. The similar result also found other authors (Amith-Romach *et al.,* 2004).This base pair is specific for *E. coli* not for others.
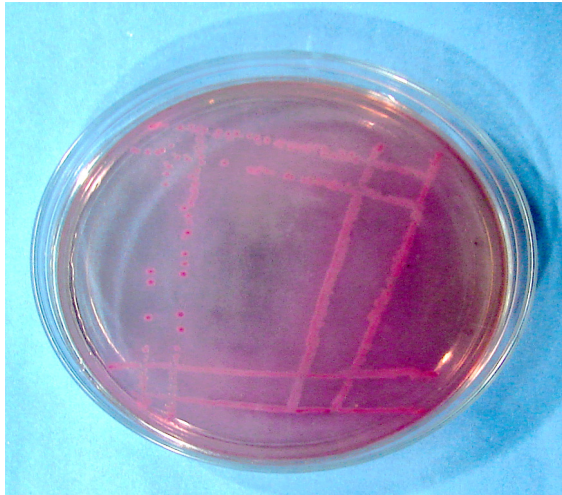
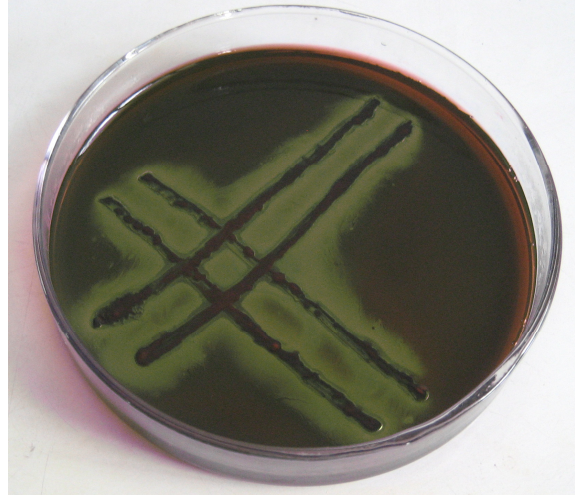Fig. 1. *E. coli* isolates showing rose pink colored colonies on MacConkey agar

Fig. 2. Isolation of *E. coli* showing characteristics greenish colored colonies with metallic sheen on EMB agar
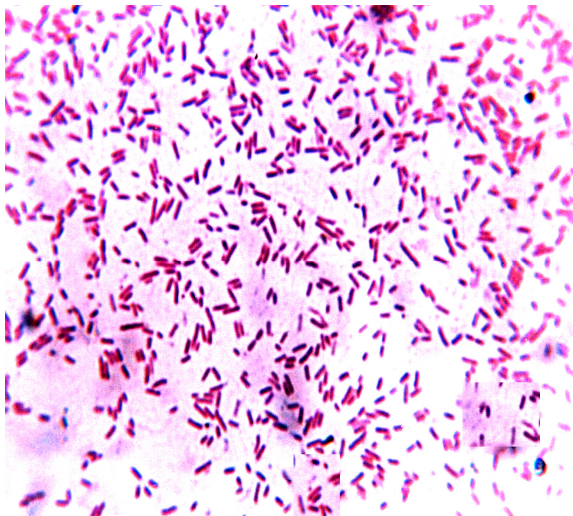

Fig. 3. Isolated *E. coli* in Gram's straining (microscopic) showing Gram negative, pink color, short rod shape organisms, arranged in single or paired (Modified Gram's Stain, ×830)

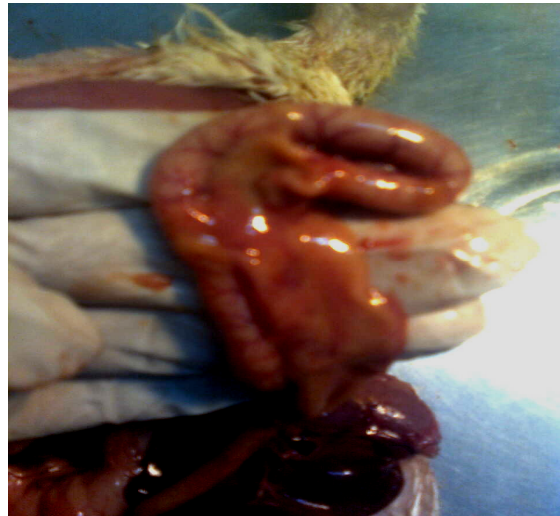Fig. 4. *E. coli* infected chickens showing the duodenum congestion, hemorrhages and excess mucus
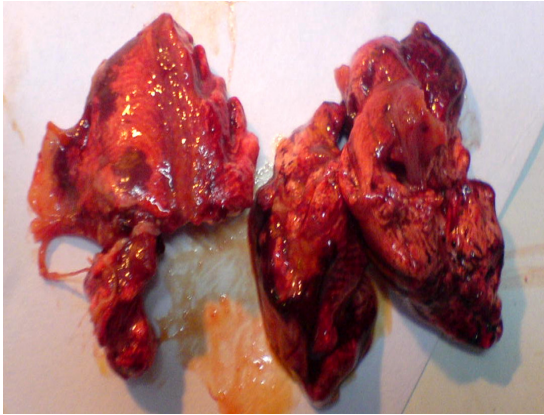
Fig. 5. Lungs of *E. coli* infected chickens showing congestion and consolidation
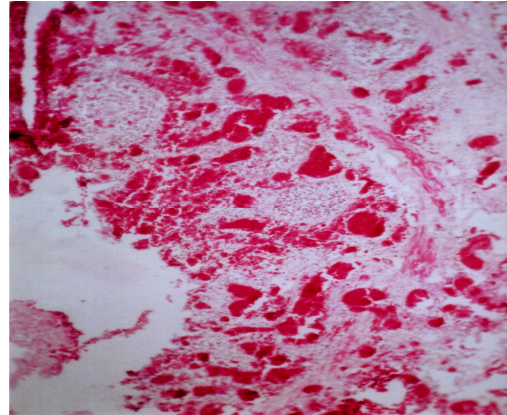

Fig. 6. The section of lung in *E. coli* infection showing severe congestion and infiltration of heterophils , macrophages and lymphocytes in the wall of the bronchus as well as In the peribronchial alveoli (H & E staining, ×83)
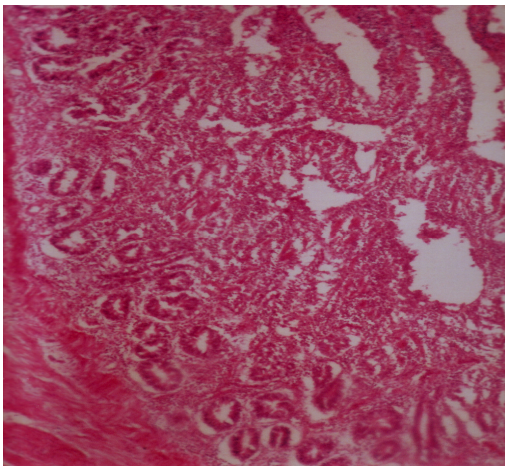

Fig. 7. The section of duodenum in *E. coli* infection showing severe infiltration of leukocytes mainly heterophils, lymphocytes and macrophages in the submucosa of the duodenal wall (H & E staining, ×83)
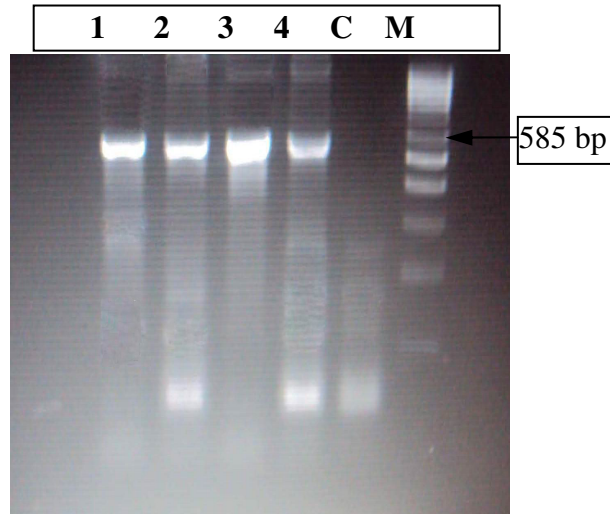

Fig. 8. Electrophoresis on agarose gel showing the 585bp PCR product (from lane 1 to lane 4) after amplified in field samples of locally isolated *E. coli* and lane C and lane M showing the negative control and marker, respectively

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