Antibacterial effect of *Nigella sativa L.* Using the Conventional Bacteriological Methods and Polymerase Chain Reaction

By

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قال تعالى

بسم الله الرحمن الرحيم

(يا أيّها الناسُ ضُربَ مثلٌ
فاستمعوا له إنّ الذين تدعون
من دون الله لن يخلقوا ذبابا ولو
اجتمعوا له وان يسلبهم الذباب
شيئا لا يستنقذوه منه ضعف
الطالب والمطلوب، ما قدروا الله
حق قدره إن الله لقوي عزيز)

صدق الله العظيم

(سورة الحج الآية73-74)

Dedication

To my family,

Friends and relatives

And to all Muslims in the world

I dedicate this work

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Abstract

E. coli strains were detected in fresh fecal samples collected from the lower large intestine and cloaca in broilers. The birds were fed on different concentrations of *Nigella sativa* crushed seeds to demonstrate antibacterial effect of the seeds .The organism was identified using conventional bacteriological methods and the polymerase chain reaction (PCR).The fecal sample were subjected to total bacterial count and then ,the identity of the nucleic acid sequence of the organism was confirmed using polymerase chain reaction-based detection assay (PCR). In the PCR assay, a pair of primers derived from uidA gene of *E. coli*, encoding glucuronidase specific for *E. coli* was used. Conventional isolation and identification is the most accurate method for detection of an active (intact) organism in fecal samples. However, this method is tedious, laborious and time consuming.The results of this study confirmed the presence of *E.coli* strains in fecal samples collected from all broilers groups .No clear effect of *N.sativa* as antibacterial agent was observed. In this work it is indicated that PCR should be used as a routine diagnostic technique for rapid detection of *E.coli* in clinical samples. It is worth mentioning that the described PCR-based provides rapid, sensitive and specific detection of both pathogenic and non-pathogenic strains of *E. coli*. Therefore, the diagnostic potential of a positive PCR result has to be interpreted with caution in light of the presence or absence of pathologic strains of *E. coli*.

ملخص الاطروحة

تم الكشف عن عترات الاشريكيه القولونيه في العيناتِ البرازيةِ التي جَمعتْ مِن القولون و المجمع في الدجاج اللاحم الذي تم تغذيته علائق مضاف لها مسحوق الحبه السوداء (لدراسة تأثيرها المُضادّ للجراثيمُ) حيث تم اضافه المسحوق لمجموعات الدجاج اللاحم بنسب مختلفه و لقد تم التعرف علي البكتيريا باستخدام الطق البكتييورلوجيه التقليديه و تقنية تفاعل سلسلة بوليمريز .

العيّنات البرازية أُخضعتْ إلى الإحصاءِ الجرثومحيث ان اثر الكمون لم يظهر لان العترات غير الممرضة تكاثرت معوضتا العدد الطبيعي للباكتيريا الامعائيه.

لقد تم الكشف عن عترات الاشريكيه القولونيه باستخدام تقنية تفاعل سلسلة بوليمريز حيث تم اشتقاق بادائين من الجين(ويد أ) الذي يشفر للانزيم جلكيورونايديز الخاص بالاشريكيه القولونيه . الطرق التقليديه للتعرف على البكتيريا د قيقة ولكن تتطب ان تكون البكتيريا حيه وهذه الطرق مرهقُه مضجرة و مستهلكة للزمن. تعتبر تقنية تفاعل سلسلة بوليمريز معين لسلسلةِ الحامضِ النووي وحسّاسة وسريعة وموثوقة بها للكشف عن عترات الاشريكيه القولونيه في العيناتِ البرازيةِ. نَتائِج هذه الدراسة اثبتت ان تقنية تفاعل سلسلة بوليمز يجب ان تستعمل كتقنية تشخيصية روتينية للكشفِ السريع عن البكتيريا في العينات السريرية و من الجديربالذكر **ان** تقنية تفاعل سلسلة بوليمريز المَوْصُوفه معيّنَ لسلسلةِ الحامضِ النووي وحسّاسَ وسريعَ لكن للعترات غير المسبّب المرض والمسبّب المرض لذاء الإمكانية التشخيصية تُترجمَ بالحذر في ضو وجود أو غيابَ عترات الاشريكيه القولونيه.

Introduction

The plant Nigella is a genre of three species: *Nigella sativa* L., *Nigella damascena* L. and *Nigella arvensis* L., commonly known as black seeds and belonging to the botanical family of Ranunculaceae. The three species have been in use in many Middle Eastern and Far Eastern countries as a natural remedy for centuries. Nigella seeds are ascribed to have many medicinal properties and used world wide in folk medicine. In Arabic countries, particularly, these seeds are considered as a real panacea, may be to religious beliefs. Consequently, many researchers have studied the antimicrobial and medicinal properties of Nigella seeds (Akhtar and Riffat, 1991 ; Rathee *et al*., 1982). Previous work on nigella seed extracts has shown that it inhibits the growth of the bacteria *Escherichia coli, Bacillus subtilis* and *Streptococcus faecalis* (Saxena and Vyas, 1986). The antimicrobial activity of *N. sativa* against several other species of pathogenic bacteria (*Staphylococcus aureus, Pseudomonas aeruginosa*) and pathogenic yeast (*Candida albcans*) has also been established (Hanafy and Hatem, 1991). In the present study different concentrations of the blessed seeds (Nigella seeds) were fed to broilers aiming to decrease the bacterial load in the feces hopefully to reduce the fecal contamination of the carcass.

Strains of *E. coli* were recognized as a cause of gastroenteritis. They are important causes of diarrhea in humans and animals in developing countries including the Sudan (Moss and Adams, 2000). Together with mycoplasma spp, the organism is also associated with chronic respiratory disease (CRD). Pollution from human and animal waste is traditionally indicated by the presence of commensal *E. coli*. Though, these organisms are essentially non-pathogenic, their presence warns of the possible

concurrent existence of pathogenic microbes (Carson *et al*, 2003). Conventional bacteriological methods are too complicated for routine use. To address these problems, different tests have been evolved for identifying the organism rapidly with a high degree of certainty.

Recently, efficient cultivation media for routine monitoring of *E. coli* in freshwater have been developed. Those methods are generally applied in a quantitative way to estimate the actual concentration of *E. coli*. Differentiation is not the main purpose for most routine applications, although there is a considerable demand for techniques for the simultaneous detection and differentiation of *E. coli* populations in natural habitat. A practical method generating a representative genetic fingerprint of population structures would enable routinely performed qualitative investigations such as the comparison of different sources of fecal emissions (Farnleitner, *et al* 1999). More recently, the polymerase chain reaction (PCR) has been proliferated because of its simplicity, rapidity, reliability, reproducibility, sensitivity and specificity for monitoring of microorganisms. PCR is a molecular- biological process that, during recent years, has been developed into a method used in virtually every area of medicine and natural sciences. PCR proved satisfactory support the conventional bacteriological methods and DNA hybridization technique (Aradaib and Ali, 2006). In the present investigation, PCR assay was evaluated for detection of *E. coli* strains in fresh fecal samples collected immediately from apparently healthy chickens.

Chapter One

Literature Review

1-1 The black cumin (seeds of *Nigella sativa*)

Nigella sativa (N. sativa) belongs to the botanical family of *Ranunculaceae* and commonly grows in Europe, Middle East, and Western Asia.

The *Nigella sativa* seeds were used in traditional medicine for long time for treatment of various illnesses affecting the respiratory system and the gastrointestinal tract especially in Islamic world. It's therapeutic use was initiated after the advent of Islam, since, Prophet Muhammad (Pbuh) mentioned its therapeutic efficacy and potential of cure. Hazard Abu Hurairah States - "*I have heard from Rasool Allah (Pbuh) that there is cure for every disease in black seeds except death and black seeds are shooneez.*" .. *Nigella sativa* seeds have high nutritive value and traditional therapeutic effects. It is an annual herbeous plant growing in western Asia and Mediterranean region for its seeds. Seeds are triangular in shape, black in color and possess a severe pungent smell, contain a considerable amount of oils .The seeds contain 40 percent fixed oil , saponin and up to 1.4 percent volatile oils(Chevallier, 1996) . The plant extract and essential oils showed abroad ranges of pharmacological effect, such as antidiabetic (Fararh *et al*., 2002; El-Dakhakhny , 2002) spasmolytic and bronchodilator (Gilani *et al.* , 2001; Boskabady *et al.* ,2004) Antioxidant(Brutis and Bucar , 2000 ; Kanter *et al.* 2003) hepatoprotective (Nagi *et al*., 1999 ; AL - Ghamdi, 2003) Antihyperlipidemic (El-Dakhakhny *et al.* , 2000) analgesic and anti-inflammatory(Hajhashemi *et al.,* 2004) Antitumor (Worthen *et al.* , 1998 ; Khan *et al*.,2003)and antiulcer effect(El-Dakhakhny *et al.* , 2000 ;

El-Abhar *et al.* , 2003). In various studies, the extracts also showed *in vitro* and *in vivo* antimicrobial (Hanafy and Hatem, 1991; Khan *et al.,* 2003) and anticestodal effects (Akhtar and Riffat, 1991). Moreover, it is reported that, the active principle of *Nigella sativa*, thymoquinone inhibited *Aspergillus niger (*AL-gabre *et al*., 2003).

It is used traditionally in Iranian community as laxative, carminative and intestinal antiprotozoal (Amin , 1990) .

The multiple use of *Nigella sativa* in the folk medicine encouraged many investigators to isolate the possible active components and to conduct *in vivo* and *in vitro* studies on laboratory animals and humans in order to understand the pharmacological actions of the baraka seeds as always Muslim societies called them.

1-1-1 The antibacterial effect of *Nigella sativa* seeds

Volatile oils of many plants are known to have antimicrobial activity (Deans *et al* . ,1992) , this activity could act as natural defense against plant pathogenic diseases .

The antimicrobial effect of *Niglla sativa* was studied extensively in the last couple of decades. The antibacterial effect of the phenolic fraction of *Nigella sativa* oil was first reported in the sixties of the last century (Topozada *et al.* , 1965). Thymoquinone, one of the active principles of *Nigella sativa* was first isolated and found to have high antibacterial activity (El-fatatry ,1975). Also in previous work on *Nigella sativa* seeds extract, has shown that it inhibits the growth of the bacteria *Escherichia coli, Bacillus subtilis* and *Streptococcus faecalis* (Saxena and Vyas, 1986) , then they also found that *Nigella sativa* seeds extract possess antibacterial synergism with streptomycin and gentamicin in addition to additive antibacterial action with spectinomycin, erythromycin, tobramycin, doxycycline,

chloramphenicol, nalidixic acid, ampicillin, lincomycin and sulphamethoxyzole-trimethoprim combination (Hanafy and Hatem,1991).Moreover the extract successfully eradicated a non-fatal subcutaneous staphylococcal infection in mice when injected at the site of infection. (Hanafy and Hatem , 1991).In other research conducted on the blessed seeds it was found that, the methanol extract of *Nigella sativa* seeds had the best antimicrobial activity and the chloroform extract had a weaker effect whereas the aqueous extract did not show any antimicrobial activity specifically against *Pseudomonas aerogenosa* and *Staphylococcus aureus* (Mashhadian and Rakhshandeh , 2005) . Furthermore other one proved that, the *Nigella sativa* oil is active against sensitive as well as multi-drug resistant strains of *Staphylococcus aureus* and may be used therapeutically in susceptible cases (Salman ,2005). More recently, the oils was tested and showed antibacterial activity against all the bacteria used in the assay (Muhammet *et al* ., 2005) .

1-2 The polymerase chain reaction

There have been a number of key developments in molecular biological techniques ; however, the one that has had the most impact in recent years is the polymerase chain reaction (PCR). One of the reasons of the adoption of the PCR is the elegant simplicity of the reaction and relative ease of the practical manipulation steps. Frequently this is one of the first techniques used when DNA is analyzed; it has opened up the analysis of cellular and molecular processes to those outside the field of molecular biology .The PCR is used to amplify precise fragment of DNA from a complex mixture of starting material usually termed the template DNA and in many cases requires a little DNA purification. It does require some knowledge of the DNA sequence information, which flanks the fragment of

DNA to be amplified (target DNA). From this information two oligonucleotides primers may be chemically synthesized , each complementary to a stretch of DNA to the 3`-side of the DNA , one oligonucleotide for each of the two DNA strands. It may be thought of as a technique analogous to the DNA replication that takes place in cells, since the out come is the same, the generation of new complementary DNA stretches based upon existing one. It also a technique that has replaced, in many cases, traditional DNA cloning methods, since it fulfils the same function, the production of large amounts of DNA from limited starting material. This achieved, however, in a fraction of time needed to clone DNA fragment. Although not without its drawback, the PCR is a remarkable development that is changing the approach of many scientists to the analysis of nucleic acid and continues to have profound impact on core bioscience and biotechnology (Wilson and walker, 2000).

1-2-1 Stages the polymerase chain reaction

The PCR consists of three defined sets of times and temperature, termed steps :(1) denaturation (2) annealing and (3)extension . Each of these steps is repeated 30 – 40 times or cycles in the first cycle the double stranded template DNA is first denatured by heating the reaction to above $90^{o}C$ .Within the complex DNA the region to be specifically amplified (target) is made accessible. The temperature is then cooled to between $40^{o}C$ to $60^{o}C$. The precise temperature is critical and each PCR system has to be defined and optimized. Reaction that is not optimized may give rise to other DNA product in addition to the specific target or may not produce any amplified products. The second annealing step allows the hybridization of the two oligonucloetide primers, present in excess, to bind to their complementary sites, which flank the target DNA. The annealed oligonucleotides act as

primers for DNA synthesis, since they provide a free 3`- hydroxyl group for the DNA polymerase. The third step, DNA synthesis or extension ,is carried out by a thermostable DNA polymerase ,most common *taq* DNA polymerase . (Wilson and walker, 2000).

DNA synthesis proceeds from both of the primers until the new strands have been extended along and beyond the target DNA to be amplified. It is important to note that, since the new strand extend beyond the target DNA they will contain region near their 3` end that is complementary to the other primer , thus if another round of DNA synthesis is allowed to take place ,not only will the original strands be used as templates but also the new strands. Most interestingly, the products obtained from the new strands will have a precise length, delimited exactly by the tow regions complementary to the primers. As the system is taken through successive cycle of denaturation, annealing and extension all the new strands will act as templates and so there will be an exponential increase in the amount of DNA produced. The net effect is to selectively amplify the target DNA flanked by the primers.

One problem with the early PCR reactions was that the temperature needed to denature the DNA also denatured the DNA polymerase. However , the availability of a thermostable DNA polymerase enzyme isolated from the thermophilic bacteria *Themus aquaticus* found in hot springs provided the mean to automate the reaction .*Taq* DNA polymerase has a temperature optimum of 72^{o}C and survives prolonged exposure to a temperature as high as 96^{o}C and so still active after each of the denaturation steps. The wide spread utility of the technique is also due to the ability to automate the reaction and as such many cycler have been produced in which it is possible

to programme–in the temperature and the times for a particular PCR reaction. (Wilson and walker, 2000) .

1-2-2 Primer design in the PCR

The key to the PCR lies in the design of the two-oligonucleotide primers. These not only have to be complementary to sequences flanking the target DNA but must not be self –complementary or bind to each other to form dimmers, since both reaction prevent DNA amplification. They also have to be matched in their G +C content and have similar annealing temperature .The increasing use of information from the internet and the sequence held in database are useful starting points when designing primer and reaction conditions for PCR and a number of software developments have allowed the process of primer design to be straightforward. In many cases this technique may be reproducibly to identify a particular organism or species. This is some times referred to as rapid amplification of polymorphic DNA (RAPDs) and has been used successfully in the detection and differentiation of a number of pathogenic strains of bacteria (Wilson and walker, 2000).

1-2-3 PCR amplification templates

The PCR may be used to amplify DNA from a variety of sources or template. It is also a highly sensitive technique and potentially requires only one or two molecules for successful amplification. Unlike many manipulation methods used in current molecular biology the PCR is sensitive enough to require very little template preparation. Extraction from many prokaryotic and eukaryotic cell may involve a simple boiling step. The PCR may also be used to amplify RNA; a process termed reverse transcriptase–PCR (RT–PCR). Initially a reverse transcription reaction that converts RNA to cDNA is first carried. This reaction is normally involves

the use of enzyme reverse transcriptase, although some thermostable DNA polymerases used in the PCR such as that from *Thermus thermophilus* (*Tth*), have reverse transcriptase activity under certain buffer condition. (Wilson and walker, 2000)

1-2-4 Sensitivity of the PCR

The enormous sensitivity of the PCR system is also one of it main drawback, since the very large degree of amplification makes the system vulnerable to contamination .Even a trace of foreign DNA such as that of contaminated in dust particles may be amplified to significant level and may give misleading result . Hence cleanness is paramount when carrying out PCR and dedicated equipment and in some cases laboratories are used. It possible that is amplified products may also contaminate the PCR, although this may be overcome by ultraviolet irradiation to damage already amplified products so that they can not be used as templates. Many traditional methods in molecular biology have now been superseded by the PCR and the applications for the technique appear to be unlimited .The success of the PCR has given impetus to the development of other amplification techniques based on thermal or non– thermal (isothermal) cycling methods. The most popular alternative method to the PCR is termed the ligase chain reaction or LCR .This operate in fashion similar to PCR but joins a thermostable DNA ligase joins set of primers together that complementary to the target DNA . Following this, a similar exponential amplification reaction takes place, producing amount of the DNA that are similar to those derived from the PCR (Wilson and walker, 2000).

1-2-5 Polymerase chain reaction is a powerful technique in medical diagnosis, forensic and molecular evolution

PCR can provide valuable diagnostic information in medicine. Bacteria and viruses can be readily detected with the use of specific primers .For example, PCR can reveal the presence of human immunodeficiency virus in people who have not mounted an immune response to this pathogen and would therefore be missed with an antibody assay. Finding of *Mycobacterium tuberculosis* bacilli in tissue specimens is slow and laborious. With PCR, as few as 10 tubercle bacilli per million human cells can be readily detected (Berg *et al* , 2001).

PCR primers specific for the recently described antimicrobial resistance-associated *Escherichia coli* clonal group A (CGA), a widespread cause of drug-resistant urinary tract infections in the United States were used for rapid and specific detection of drug resistant bacteria (James *et al* 2004). Also The presence of a hemolysin-encoding gene, *elyA* or *hlyA*, from Shiga toxin-producing *Escherichia coli* (STEC) was detected by PCR (Anselm *et al* 1998).

1-3 The Detected Bacteria *Escherichia coli* (*E.coli*)

The *E.coli* belongs to the genus Escherichia which is composed of several species but only the *E.coli* is an important pathogen of animals (Dweight and Yuan ,1999). This species , the major facultative gram-negative species, comprises the normal flora of the GIT and may be the cause of septicemic diseases of foals ,calves, piglet, puppies and lambs , enterotoxigenic diarrhea in new born farm animals; and of edema disease in pigs . It may also be opportunistic in almost all animal species (e.g. in urinary tract diseases, abscesses and pneumonia) .Among the most important disease caused by the *E.coli* is collibacilloses of fowl which is economically important disease caused by invasive strains of *E.coli* (Dweight and Yuan ,1999) .

Strains of *E. coli* were recognized as a cause of gastroenteritis. They are important causes of diarrhea in humans and animals in developing countries including Sudan (Moss and Adams, 2000). Together with Mycoplasma spp, the organism is also associated with chronic respiratory disease (CRD). Pollution from human and animal waste is traditionally indicated by the presence of commensal *E. coli*. Though, these organisms are essentially non-pathogenic, their presence warns of the possible concurrent existence of pathogenic microbes (Carson *et al,* 2003). Conventional bacteriological method is too complicated for routine use. To address these problems, different tests have been evolved for identifying the organism rapidly with a high degree of certainty.

Chapter Two

Materials and methods

2-1The experimental birds

One-day-old chicks (Lohwman broilers) were purchased from Private Company in Khartoum and brought to the laboratory of the department of Biochemistry, Faculty of Veterinary Medicine, University of Khartoum, Sudan. The experimental birds were subjected to visual inspection and clinical examination to exclude the possibility of infection with contagious or infectious avian diseases. The experimental birds were reared together in an opened system and were fed on balanced ration (table 1) containing different concentrations of crushed black seeds with free access to water. The birds were allotted randomly into 3 groups (each group contains 33 birds and was divided into two subgroups as replicate). Each one of the groups was provided with different percentage of *Nigella sativa* crushed seeds per ration as described below .

Three graded level of *Nigella sativa* crushed seeds (0.75% , 0.25% and 0.0%) were added replaced (w/w) to the basal ration of composition in table (1) designated as rations A , B and C respectively. Each ration was fed to the subjected group (The crushing and addition were taken place at the same day to protect volatile content of the seeds) .The birds were vaccinated against Gumboro disease at the second week and repeated at the fourth week of age together with Newcastle disease vaccine which was also administered at the fourth week of age.

2-2. Housing and Management

The experiment was carried out in an open mesh sided, deep litter poultry house at the department of Biochemistry, faculty of Veterinary Medicine. The house subdivided in to 6 rooms made of wire netting.

Enough space for work was left. The house was cleaned, washed, and disinfected using formalin and

Table (1): Percent composition of the basal experimental ration

The composition	Percentage
crude protein	23%
Crude fiber	3.2%
Crude lipid	4.25%
Lysine	1.25%
Methionine	0.41%
Calcium	1%
Total phosphorous	0.5%

The energy 3000 Kg

malathione, each pen floor was covered with enough wood shaving with allocation of two feeders and waterers. The eastern and western sides were covered in the late afternoon and early evening to keep the temperature and ventilation suitable. Strict sanitation and biosecurity measures were followed, unnecessary visitors were not allowed on to the house, all animal including wild birds, pets and rodents were kept away. Footbath filled with fresh disinfectant at the entrance of the house was kept. A 100-watt bulb per pen was used for artificial lighting through the evening time.

2-3 Collection of fecal samples

In the fifth and sixth weeks of life randomly selected two birds from each cage were slaughtered and post mortem was carried out (full precautions was taken to prevent contamination). Using fresh sterile forceps , scissors and scalpels, fresh fecal samples were collected directly from the cloaca and the lower large intestine to prevent contamination. Fecal samples were preserved in fifty ml plastic containers and submitted to diagnostic laboratory for further investigations including bacterial isolation and identification as well as Polymerase chain reaction (PCR).

2-4 Bacteriological Investigation

2-4-1 Bacteriological Media

2-4-1-1 MacConkey agar (biomark)

constituents (gm/litre)

Peptic digestion of animal tissues 20, lactose 10, bile salts 1.5,Sodium Chloride 5.0, Crystal violet 0.001, Neutral red 0.05 , Agar 15 .

Final pH at 25^{o}C 7.2 .

Method

51.55 grams were suspended in 1000 ml distilled water, then the medium was boiled and dissolved completely .The medium was sterilized by autoclaving at 15 Ibs pressure for 15 minutes .The over heating was avoided then cooled to 40-45^{o} C and poured into sterile Petri plates. The fecal samples were grown on MacConkey medium as selective and differential medium to execlude non-lactose fermenter of the family Entrrobactereiaceae. Lactose is included as a fermentable carbohydrate with a pH indicator, usually neutral red. Strong acid producer like *Escherichia* ,*Klebsiella* and *Enterobacter* produced red colonies.

2-4-1-2 Nutrient agar

Dehydrated nutrient agar (Oxoid Ltd England) was prepared according to the manufacturer instruction. This medium consists of yeast extract, sodium chloride and agar. Twenty eight gram of the powder were dissolved in 1 liter of distilled water by boiling .The pH was adjusted to 7.4 and then the medium was sterilized by the autoclave (121^{o}C for 15 minutes) cooled to 50^{o}C−55^{o}C and then distributed to sterile Petri dishes 20 ml in each dish.

2-4-1-3 Peptone water

Peptone water constituents

Peptone	10 gm
NaCl	5 gm
Water	1000ml

The solids were dissolved by heating in the water, the pH 8.0−8.4 was adjusted and then the solution was boiled for 10 for minutes then it was filtered, the pH adjusted to pH 7.2 −7.4.The medium was distributed in 5ml into test tubes then sterilized at 115^{o} C for 20 minutes.

2-4-1-4 Peptone water sugar

The method of preparation would depend on the indicator. The reaction of 900 ml peptone water was adjusted to pH 7.1−7.3 so that the addition of 10 ml of Andrade's indicator would bring it to pH 7.5, then it was sterilized at 115 ^{o} C for 20 minutes. The medium was pink when hot but the color was faded on cooling.

Then 10−5 gram of the sorbitol sugar was dissolved in 90ml of water and steamed for 30 minutes. Aseptically this was added to the sterile peptone water with indicator, and then distributed in sterile test tubes.

2-4-2 Bacteriological procedures

2-4-2-1 Determination of the viable organisms in the sample

The procedure of Miles and Misra was used .One Gram of feces was suspended into a sterile diluent (normal saline). The suspension was then 10 fold serially diluted.

The dilution tube were first labeled as 10^{-1} , 10^{-2} , …..,10^{-7} then one ml of the sample suspension was used to inoculate the first dilution tube using micropipettes with sterile tip .Then with a fresh sterile tip the content of the first dilution tube was mixed before one ml was withdrawn to inoculate the second dilution tube . The process of serial dilution was repeated till tube number 7(ie 10^{-7}).

2-4-2-2 Cultivation of the diluted samples

Dilutions number 10^{-4},10^{-5}, 10^{-6}wereused to inoculate MacConkey agar.Plates were first dried in the incubator at 37^{o}C for 30 minutes .Two plates were used for each dilution .Plates were labeled and the 20 ml of each dilution was used to inoculate the middle of the plate. The plate was moved gently to spread the inoculum. Inoculated plares were left for half an a hour on the bench to allow absorption of the inoculum . Plates were then incubated aerobically at 37^{o}C for 24 hours .After incubation plates showing

between 50-100 colonies were used for count . The total number of the colonies of the suitable dilution was divided by two to abtain the mean (duplicate plates).Then the mean was multiplied by 50 by the reciprocal of the dilution .The resuit was expressed as CFU/ml.

2-4-2-3 Isolation and Purification of *E.coli*

Gram stain was performed for red colonies, when gram negative bacteria, appeared as short rods or cocci, then they were identified by indol production and sorbitol test and then the identity of the nucleic acid of the organism was identified usin PCR based detection assay.

2-4-2-4 Identification of *E.coli*

Biochemical Bacteriological tests

All bacteriological biochemical tests were performed according to Barrow and Felthman , (1993).

2-4-2-4-1 Indol production test

Reagents

Peptone water

Indol kovac's reagent (Ingredient per liter)

Dimethylaminbenzinaldehyde 50.0gm

Hydrochloric acid 37 ٪250 ml

Amyl alcohol 750 ml

The aldehyde was dissolved in alchohol by gentle warming in water bath about (55^{o}C −50^{o}C) then cooled and added to acid with care, protected from light and stored at 4^{o} C.

Equipment

Test tube containing peptone water

Dropper to drop Kovacs reagent.

Bottle containing Kovac's reagent.

Bacteriological loop.

Principle

Indol is one of the degradation products from the metabolism of the amino acid tryptophan. Bacteria that possess the enzyme tryptophanase are capable of cleaving tryptophan and there by producing the indol, pyruvic acid and ammonia. Indol can be detected in tryptophan test medium by observing the development of a red color after adding a solution Dimethylaminbenzinaldehyde (kovac's reagent**)** and the negative result detected when no color changed upon the addition of kovac's reagent

Procedure

The Peptone broth is lightly inoculated by the bacteriological loop with the test organism and incubated 24-48 hours at 35^{o}C. Four to five drops of Kovac's reagent were added to the tube was shaken gently. The development of red color indicates indol production.

2-4-2-4-2 **Sorbitol test**

Reagents

Peptone water
Peptone water sugar

Preparation of Andrade's indicator.

Acid fuchsin	5 gm
Distilled water	1ooo ml
N. NaOH as required	150 − 180 ml

The acid fuchsin was dissolved in distilled water and 150 ml of alkali solution was added, mixed and allowed to stand at room temperature with frequent shaking for 24 hours, the color was change from red to brown. Subsequent addition of alkali was made until a straw − yellow color is ultimately attained with minimum addition of alkali.

To the test final indicator, 1% of the indicator solution to peptone water at pH 7.2 was added and mixed thoroughly.

Equipment

Test tube containing the peptone water sugar (sorbitol).

Bacteriological loop for inoculation.

Principle

Firstly the bacteria have to possess the enzyme**,** Sorbitol Dehydrogenase to covert the sorbitol to fructose in the presence of nicotinamide adenine dinucleotide (NAD), then the fructose may be phosphorylated to form fructose 6−phosphate, catalyzed by the same enzyme, hexokinase that accomplished the phosphorylation of glucose, another enzyme, fructokinase can perform the same mission (Robert *et al.* , 2003)

Then the fructose 6−phosphate undergoes anaerobic glycolytic pathway to form Pyruvic acid and the chemical consequence by which glucose is converted to pyruvic acid is known as Embden − Mayerhof pathway (Elmer *et al.*, 1997) glucose fermentation by *Escherichia coli* result in production of large quantities of acetic acid , lactic acid and formic acid , with a marked drop in the pH of the test medium ,This is detected by the indicator added to the medium (Elmer *et al.*, 1997) .

Procedure

The sugar medium was inoculated with bacteria grown on the nutrient agar, incubated overnight and then examined daily for 7 days. Acid production (positive result) was indicated by the development of pink color in the medium due to the drop in the pH which changes the color of the indicator (neural red) whereas the negative result was indicated by unchanged color of the medium.

2-5 Nucleic acid techniques

2-5-1 Extraction of Bacterial DNA

Using a bacteriological loop, pure colonies of isolated strains of *E. coli* were transferred to epindorf tube containing 300 µl of double distilled water. The colonies were resuspended in the distilled water by vortexing. DNAs were extracted from bacterial samples by boiling the tubes in a water bath at 100^{o} C for 30 min**.** The epindorf tubes were then centrifuged at 12,000 RPM for 15 minutes and the supernatant containing DNAs was transferred to new tubes and stored at -20^{o} C until used for PCR amplification.

The bacterial DNA concentration was determined by spectrophotometer at 260-wave length. Five microliters of the suspended nucleic acid was used in the PCR amplification.

2-5-2 Primers selection

A pair of oligonucleotide primers (P1 and P2) was derived from a highly conserved region of nucleotide sequences of the uidA gene which codes for glucuronidase specific for *E. coli* and Shigella (Juck *et al*., 1996). Primers 1 and 2 (P1 and P2) were selected for the synthesis of specific PCR product. P1 (5'-ATC ACC GTG GTG ACG CAT GTC GC-3') included 23 bases of the positive sense strand. P2 (5'-CAC CAC GAT GCC ATG TTC ATC TGC-3') included 24 bases of the complementary strand. The PCR using primer P1 and P2 would result in a 486 bp PCR product. For nested amplification, two pairs of internal primers were designed (P3 and P4). Oligonucleotides P3 (5'-TAT GAA CTG TGC GTC ACA GCC-3') and P4 (5'-CAT CAG CAC GTT ATC GAA TCC-3') were used in the nested PCR to amplify a 186-bp fragment from the primary PCR. All primers were synthesized on a DNA synthesizer (Milligen/Biosearch, a division of MilliporeBurlington, MA, USA) and purified using oligo-pak

oligonucleotide purification columns (Glen Research Corporation, Sterling, Virginia, USA.).

2-5-3 Polymerase chain reaction (PCR)

A stock buffered solution containing (250 micro liter (µl) 10X PCR buffer, 100 µl of 25 mM Mg $Cl_{2,}$ 12.5 µl of each dNTPs (ATP, TTP, GTP and CTP) at a concentration of 10mM) was prepared in 1.5 ml tube. The primers were used at a concentration of 20 picograms. Double distilled water was added to bring the volume of the stock buffer solution to 1.5 ml. For each PCR amplification, 5.0 µl of the target DNA and 2 ul of primers was added to 42 µl of the stock solution in PCR tubes and mixed by vortexing. 1.0 µl of Taq DNA polymerase (Perkin Elmer) was used at a concentration of 5.0 units. All PCR amplification reactions were carried out in a final volume of 50 µl. The thermal cycling profiles were as follows: a 2-min incubation at 94^{o}C, followed by 35 cycles of 94^{o}C for 1 min, 57^{o}C for 30 sec and 72^{o}C for 45 sec. A final incubation at 72^{o}C for 10 min was carried out to ensure complete synthesis of the expected PCR products.

Thermal profiles were performed on a Techne PHC-2 thermal cycler (Techne, Princeton, NJ.)(Figure1). Following amplification, 10 µl from each PCR reaction containing amplified product were loaded onto gels of 1.0% SeaKem agarose (FMC Bioproduct, Rockland ME)and electrophoresed (figure 2.) The gels were stained with Ethidium bromide and the specific 486 bp PCR products were identified following visualization under UV light (figure 3) .

2-5-4 Nested polymerase chain reaction (nPCR)

For nested PCR, 5 µl of the amplified product were added to 42 µl of the second master mixture. The master mixture for the nested PCR contained

the following: 2 µl of primers P3 and P4 at a concentration of 20 uM/liter, 42 µl of the PCR stock solution, 1.0 µl of Taq DNA polymerase (Perkin Elmer) was used at a concentration of 5.0 units. All PCR amplification reactions were carried out in a final volume of 50 µl. The nested PCR amplification was performed with an initial denaturation step at 95°C for 2 min; 35 cycles at 95°C for I min, 57°C for 30 sec, and 72°C for 45 sec; and a final elongation phase at 72°C for 10 min.

The product of the nested PCR was electrophoresed with 3 µl of loading buffer (0.25 g of bromphenol blue and 40 g of saccharose dissolved in 100 ml of

distilled water) through a 2% agarose gel at 80 V for 45 min. Molecular size markers (Promega, Madison, Wis.) were run concurrently. The gel, stained with ethidium bromide (0.5 µg/ml), was examined under UV light for the presence of a 186-bp nested PCR product and photographed for documentation.

Figure1.

The thermal cycler for the DNA amplification.

Figure 2.

The Agarose Gell Electrophoresis

Figure 3.

UV Transilluminator for gel documentation

Chapter Three

Results

3-1 The bacterial count

The bacterial counts of the fecal samples collected from broilers at the fifth and the sixth week of life were shown in table (2).

3-1-1 The bacterial count at week five

There was no statistical significant difference between the groups fed *Nigella sativa* on the higher concentration of 0.75% or on 0.25% *Nigella sativa* per ration and also no significant difference between them and the group which kept as control.

This finding was true when the samples were compared at week five and week six. But a highly significant (P 0.05) different was observed for each group when samples from week five were compared to the samples from week six. Though there was no statistical significant difference between the count in the different groups but group B that fed on 0.25% *N.sativa* presented very low bacterial count when compared to other groups. This was very clear in the samples taken at week six, where the control showed very high count compared to the Nigella fed groups.

3-2 MacConkey agar

When the fecal samples collected from the broiler chicken were cultivated on the MacConkey's media they appeared as confluent pink colonies which was evident for the *E.coli.*

3-3 Gram stain

Gram-negative bacteria morphology resembling those of *E.coli* was encountered in stained smear (Gram-stain smear) of purified colonies in all fecal samples.

3-4 the bacteriological biochemical test

3-4-1 The sorbitol test

The sorbitol was fermented by the bacteria isolated from the feces of the three groups so the result can be considered as positive, The result was shown in table (3).

3-4-2The Indole production test

The tested bacteria isolated from the feces of the three groups could produce indole so the test can be considered as positive The result was shown in table (4).

3-5 The polymerase chain reaction

The described PCR-based assay afforded sensitive and specific confirmation of *E. coli* from all fecal samples colleted from chickens used in this study. The specific 486 bp PCR product was visualized on ethidium bromide-stained gel from DNA of *E.coli* (Figure 6). Using the internal primers (P3 and P4), the nested PCR resulted in amplification of a 186 bp PCR product. The nested 186 bp PCR product was detected from smaller amount of *E.coli* DNA target (Figure 4and 5). Using crude extracted *E.coli* DNA target, the 186 bp specific PCR products was detected in the all *E.coli* strains isolated from fecal samples (Figure 8). Negative control samples including samples with out DNA target and nucleic acid-free water failed to demonstrate the 486 bp primary specific or the 186 bp nested PCR products (Figure 6 and7). All bacterial isolates, which were PCR positive, were also found culture positive as confirmed by conventional isolation methods.

Table 2

The bacterial count (colony forming unit per ml CFU/ml) result in the fifth and the sixth week of life. (Mean ± standard error).

The sample	The fifth week ± Std.Error	The sixth weeks ± Std.Error
A	$4.3 \times 10^{7} \pm 2.5 \times 10^{7 a A}$	$3.3 \times 10^{8} \pm 25 \times 10^{7 b B}$
B	$1.48 \times 10^{7} \pm 5.7 \times 10^{7 a A}$	$1.359 \times 10^{8} \pm 6.7 \times 10^{7 b B}$
C	$2.5 \times 10^{7} \pm 6.5 \times 10^{5 a A}$	$6.53 \times 10^{8} \pm 20.1 \times 10^{7 b B}$

Means within the same column having different small letter were significantly different.

Means within the same row having different capital letters were significantly different.

A = the group of birds fed on 0.75% *Nigella sativa* per ration.

B= the group of birds fed on 0.25% *Nigella sativa* per ration.

C= the group of birds fed on 0.00% *Nigella sativa* per ration (control)

Table (3): The sorbitol test result

The sample	Fecal sample at day 35 of age	Fecal sample at day 45 of age
A1	+ve	+ve
A2	+ve	+ve
A3	+ve	+ve
Aw	+ve	+ve
B1	+ve	+ve
B2	+ve	+ve
B3	+ve	+ve
Bw	+ve	+ve
C1	+ve	+ve
C2	+ve	+ve
C3	+ve	+ve
Cw	+ve	+ve

Table (4): The indol production test result

The sample	Fecal sample at day 35 of age	Fecal sample at day 45 of age
A1	+ve	+ve
A2	+ve	+ve
A3	+ve	+ve
Aw	+ve	+ve
B1	+ve	+ve
B2	+ve	+ve
B3	+ve	+ve
Bw	+ve	+ve
C1	+ve	+ve
C2	+ve	+ve
C3	+ve	+ve
Cw	+ve	+ve

A1 , A2 ,A3 = birds from the group which given 0.75% *Nigella sativa* per ration

Aw = The drinking water taken from the group which given 0.75% *Nigella sativa* per ration.

B1, B2, B3 = birds from the group which given 0.25% *Nigella sativa* per ration.

Bw = The drinking water taken from the group which given 0.25% *Nigella ativa* per ration.

C1, C1, C2 = birds from the group which given 0.0% *Nigella sativa* per ration (ie control)

Cw = the drinking water taken from the group which given 0.0 % *Nigella ativa* per ration.

Figure 4

Sensitivity of the polymerase chain reaction for detection of the primary 186-bp PCR product from *E.coli* DNA.

Visualization of the 186-bp specific- PCR product on ethidium bromide-stained agarose gel from *E. coli* DNA extracted from fecal samples of apparently healthy chickens. Lane MW: molecular weight marker (1 Kb ladder); lane 1 nucleic acid free water.; lanes 2-6*: E.coli* DNA , 100 ng ,10 ng , 1ng , 100pg , 10 pg respectively.

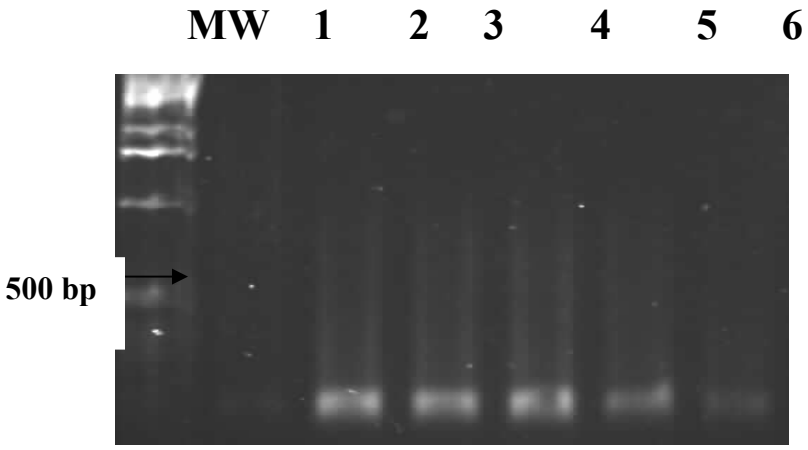


Figure 5.

Sensitivity of the nested polymerase chain reaction (nPCR)for detection of the nested *E.coli* 186 bp PCR product .

Visualization of the 186-bp specific- PCR product on ethidium bromide-stained agarose gel. Lane MW: molecular weight marker ; lane 7:nucleic acid free water ; lanes 1 *E.coli* DNA 1pg ; lane 2 *E.coli* DNA 10 pg ; lane 3 *E.coli* DNA 100pg ; lane 4 *E.coli* DNA 1 ng ; lane 5 *E.coli* DNA 10ng ; lane 6 *E.coli* DNA 100 ng .

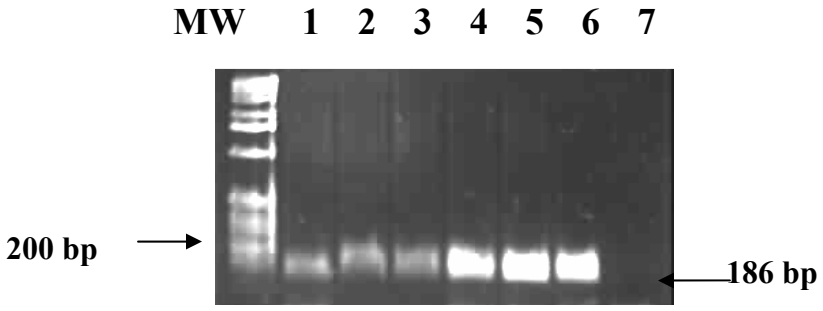


Figure 6.

Visualization of the 486-bp specific-*E.coli* PCR product on ethidium bromide-stained agarose gel from DNA of E.coli strains. Lane MW: molecular weight marker; Lane 1 and 3: *E.coli* DNA; Lane 2: Negative control.

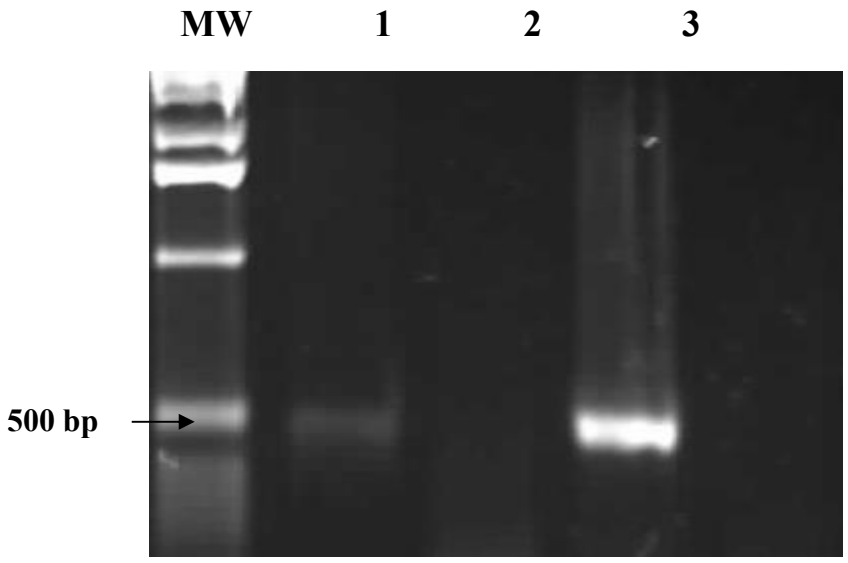


Figure 7.

polymerase chain reaction (PCR) amplification of E.coli DNA showing the specific 486 bp PCR product and the nested 186 bp PCR product. Amplification product was not detected from DNA from disttled water and nucleic acid-free water. Lane MW: molecular weight marker; Lane 1: nested 186 bp PCR product from E.coli DNA. Lane 2: Primary 486 bp PCR product; Lane 3: nucleic acid-free water (negative control).

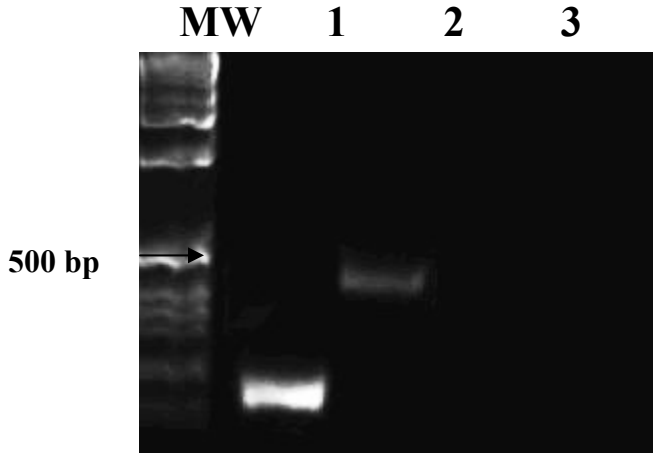
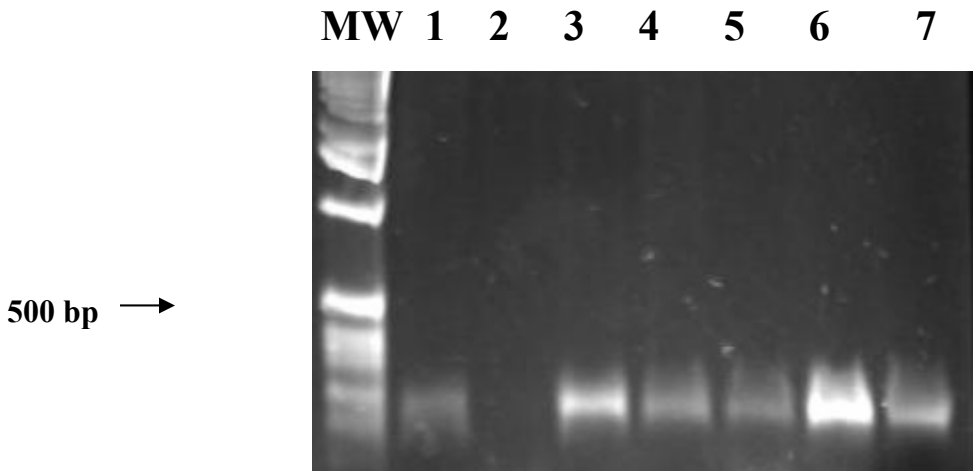


Figure 8.

Visualization of the 186-bp specific- PCR product on ethidium bromide-stained agarose gel directly from fecal samples suspected to contain *E.coli*. Lane 1: *E.coli* DNA positive control; Lane 2: negative control; Lane 3-7: fecal samples containing *E. coli* DNA.



Chapter Four

Discussion

4-1 The antibacterial effect of *Nigella sativa*

The antibacterial effect of *Nigella sativa* was studied extensively in the last few years (Hanafy , Hatem ,1991; Mashhadian and Rakhshandeh , 2005) .The ultimate goal of this research was to investigate the effect of the crushed blessed seeds on the bacterial load (Enterobacteriaceae) in the large intestine of broiler chickens as was mentioned earlier (The crushing and addition were taken place at the same day to protect volatile content of the seeds) .

Result showed that there was no statistical significant difference between the samples which were collected on day 35 of bird life for all groups, i.e. there was no significant difference between the group that was kept as control (fed on 0% of *Nigella sativa* per ration) and the groups that fed 25% and 75% of *Nigella sativa* per ration , Moreover there was no significant difference within the group, this was also true for samples that were collected on day 45 of bird life, however , there was significant difference between the samples of day 35 and the sample of day 45 of bird life which might be due to the administration of Enrofloxacin in water because the birds were infected (at day 25 of life) with pathogenic strain of *E.coli* where bacteriological identification and sensitivity test were performed for the birds and the result showed that Enrofloxacin was the drug of choice .

Hanafy and Hatem (1991) observed antimicrobial activity of diethyl ether extract of the plant in the concentration of 25-400 mcg/disk against *E. coli* but they conduct the research invitro. Mashhadian and Rakhshandeh (2005) demonstrated the antimicrobial activity of methanol extract of *N. sativa*

seeds against other organisms but an aqueous extract showed no antimicrobial activity and their research was also invitro. In the present work though difference between the groups were not significant but the control showed very high value compared with *N.sativa* fed groups specially the group that fed 0.25%.

Dweight and Yuan , (1999) mentioned that , the normal habitat of *E. coli* is the large intestine . In the present work fecal samples were collected from the lower large intestine and cloaca , so presence of *E.coli* is expected in that location in apparently healthy birds , and if the antimicrobial effect of the blessed seeds had prevailed against the pathogenic strains , the non pathogenic strains will dominate , compensating the normal counts of *E.coli* so the result can agree with Hanafy and Hatem ,(1991) and the result of Mashhadian and Rakhshandeh , (2005) .

The result showed the cultivated bacterial colonies appear on the MacConkey agar after 24 hours at 37^{o} C as red or pink colonies which is typical *E.coli* colonies (Oxoid Ltd England) .

4-2 The bacteriological biochemical test

The result showed that the performed bacteriological tests (sorbitol tset and indol production test) were positive result i.e. the sorbitol was fermented and the indol was produced by all tested bacterial sample, these test are known to be positive for *E.coli* indicating the presence of the organism in the samples (Elmer *et al.*, 1997)

4-2 The polymerase chain reaction

Application of PCR for detection and identification of bacterial organisms have been described by several workers. The described nested PCR assay reproducibly and specifically detected *E.coli* DNA from fecal sample. The

specific 486 bp PCR products or the nested 186 bp PCR product, visualized on ethidium bromide-stained agarose gel, were obtained from all *E.coli* strains.

The sensitivity of the PCR protocol was comparable or even more sensitive than conventional bacteriological procedure. Our described PCR assay is million times more sensitive than the hybridization assays using cDNA probe, where at least a few nanograms of the bacterial DNA are required to produce a positive hybridization signal. Temperature and time for denaturation, primer annealing and extension, enzyme and $MgCl_2$ concentration, and number of cycles of the three temperatures per time segments were very important for maintaining sensitivity and specificity of the PCR reaction. Thirty cycles of amplification were used as standard PCR procedure. The time required for PCR assay was approximately 4 hours which include one hour for DNA extraction, two hours for the PCR amplification and one hour for electrophoresis and running of the agarose gels. Excellent correlation of results from the primary and nested amplification was obtained using this PCR protocol. Nested could be used to confirm the specificity of the 486 bp primary amplified product and to increase the sensitivity of the PCR assay particularly, when the concentration of the bacterial DNA in the suspected sample is too small .

The time required for this nested PCR protocol is consistently 4 hours, which means that the described nested PCR protocol could be done with in the same working day. There was variation in the intensity of the PCR bands when exposed under ultraviolet light. This could be due different concentration of extracted bacterial DNA used in this study. The result of this study indicates that the described PCR protocol has the potential to detect *E.coli* in at a very small number of organisms. Therefore, it is not uncommon to obtain PCR-positive, but bacterial isolation negative, results from the same environmental

sample as only a few numbers of *E. coli* target DNA copies are needed for PCR amplification.

The *E.coli* PCR can replace the need for the lengthy cumbersome bacterial isolation procedures. The rapidity, sensitivity and specificity of the nested PCR assay would greatly facilitate detection of *E.coli* in the suspected samples particularly, those associated with fecal contaminations. Negative and positive controls should be included in each PCR reaction to estimate the lower limit of specificity and the higher limit of sensitivity. Further studies are currently under way to develop and evaluate a PCR protocol, which could be used for simultaneous detection and differentiation of pathogenic and non-pathogenic strains of *E. coli* from clinical samples or environmental sources.

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