Introduction

With amplified antibiotics usage over current decades, there has been an emerging alarm about the speeded development of antibiotics resistance in the environment. This development poses several public health worries, such as, the higher frequency of multiple drug resistant bacteria both in the environment and in clinical settings. The presence of antibiotic-resistant bacteria has reached epidemic proportions in recent years. The spread of antibiotic-resistant bacteria in the environment is dependent on the presence and transfer of resistance genes among microorganisms, and selection pressure to keep these genes in a population(Cizman, 2003).

Antimicrobials are often used to treat and prevent infectious disease or to promote growth. Many of these antimicrobials are identical to or closely resemble drugs used in humans. Antimicrobial resistance has emerged in commensal bacteria (e.g., *Escherichia coli, Enterococci spp.*), zoonotic entero-pathogens (e.g., *Salmonella spp., Campylobacter spp.*), and bacterial pathogens of animals (e.g., *Pasteurella, Actinobacillus spp*.), but the prevalence of resistance varies. Antimicrobial resistance appears from the use of antimicrobials in animals and the subsequent transfer of resistance genes and bacteria among animals and animal products and the environment. To slow the development of resistance, some countries have restricted antimicrobial use in feed, and treatment of diseased animal. Alternatives to growth-promoting and prophylactic uses of antimicrobials in agriculture include improved management practices, wider use of vaccines, and introduction of probiotics. Monitoring programs, careful use guidelines, and educational campaigns provide approaches to minimize the further development of antimicrobial resistance. These programs usually monitor indicator bacteria such as *Escherichia coli* (Cizman 2003; Aarestrup 2004; Li *et al.*, 2010).

E. coli is commonly found in human and animal intestinal tracts and, as a result of faecal contamination or contamination during food animal slaughter, is often found in soil, water, and foods. A number of *E. coli* strains are recognized as important pathogens of Colibacillosis in animals can cause severe human diseases such as haemorrhagic colitis and haemolytic uremic syndrome (Riley *et al,*. 1983; Chansiripornchai, 2009; Ferens and Hovde, 2011). The treatment of illnesses caused by this bacterium often requires antimicrobial therapy. The decision to use antimicrobial therapy depends on the susceptibility of the microorganism and the pharmacokinetics of the drug for achieving the desired therapeutic concentration at the site of infection and thus clinical efficacy (McKellar *et al.,* 2004). However, veterinary practitioners have a limited choice of antimicrobials for use in the veterinary medicine, due to antimicrobial resistance issues and human health concerns. Moreover, the repeated and unsuitable use of antibiotics has led to an increasing rate of antimicrobial resistance (Mooljuntee *et al.,* 2010).

Tetracyclines are antibiotics and acts to inhibit bacterial growth by interfering with protein synthesis. The emergence of bacterial resistance to these antibiotics has nowadays limited their use. Three different mechanisms of tetracycline resistance have been identified so far: tetracycline efflux, ribosome protection, and tetracycline modification (Arzese *et al.,* 2000; Blake *et al.,* 2003). Tetracycline efflux is achieved by an export protein from the major facilitator super family (MFS) which contains 12 transmembrane fragments (TMS) in Gram-negative bacteria and 14 in Gram-positive bacteria. Ribosome

protection is mediated by a soluble protein which shares homology with the GTPases participating in protein synthesis by elongation factor-Tu (EF-Tu) and EF-G. The third mechanism involves a cytoplasmic protein that chemically modifies tetracycline, a reaction that takes place only in the presence of oxygen and NADPH and does not function in the natural host. The most common resistance mechanism in Gram-negative bacteria is the energy-dependent efflux pump system which is encoded by the genes tetA, tetB, tetC, tetD, and tetG, with tetA and tetB genes being the most frequently described.

In Iraq, antimicrobials are freely available and used for human and animal without prescription from the specialist and estimates of its used vary widely. This misuse has led to increasing in the number of bacteria resistant to multiple antimicrobial agents involved in clinical infections. This situation is threatening the effectiveness of even the most reliable antibiotics used to treat bacterial infections (Wegener *et al.,* 1999). The problems of resistant bacteria are particularly more serious in hospitals and nursing homes where patients are treated for acute or chronic infective conditions (Roberts, 1996). Antibiotic-resistant bacteria transmission often occurs in communities by person-to-person transfer, through contaminated food, unsafe drinking water, or by insects. Resistance can mean that people infected with such bacteria do not respond to conventional drugs and, if no other treatment options are available, must depend on their immune system to overcome the disease (Wegener *et al.,* 1999; Chopra and Roberts, 2001).

Due to the excessive use of tetracycline and oxytetracycline in veterinary practice in Iraq for more than 3 decades for treatment of diseased animals. The veterinary daily clinical observations revealed that oxytetracycline is routinely misused and abused and its efficacy was decreased in the treatment of acute and chronic clinical cases. Veterinarians in practice are searching for another and a new antimicrobial products nowadays. Recently, molecular techniques, especially polymerase chain reaction (PCR), have been widely used to study antimicrobial resistance genes.

Aims of study

There is a paucity of information on the molecular studies regarding oxytetracycline resistance bacteria in Iraq. Therefore, this study was design to

(1) identify the presence of drug resistant bacteria
(2) identify the prevalence of tetracycline resistance genes tet (A) in isolated in vitro resistance *E. Coli*.

Review of literature

History of Antibiotic Development

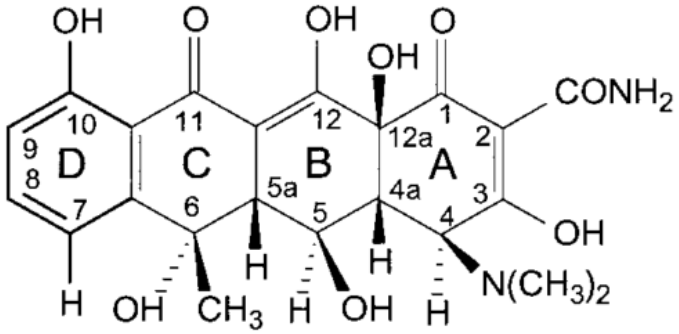
Antimicrobial drugs have generally been classified into two categories, one includes the synthetic drugs, such as the sulfonamides and the quinolones, and the second, antibiotics, synthesized by microorganisms. In recent years, increasing numbers of semi-synthetic drugs have been developed, which are chemical derivatives of antibiotics, thereby blurring the distinction between synthetic and natural antibiotics. In earlier times, plant products were sometimes used successfully in the treatment of diseases, but neither doctors nor patients knew the basis for the action of these therapeutic agents. Many early medicines were used to cure protozoan diseases, rather than bacterial diseases. As early as 1619, it was known that malaria could be treated with the extract of cinchona bark (quinine) and that amoebic dysentery could be treated with ipecacuanha root (emetine) (Garrod and O'Grady, 1971; Greenwood, 2000). Only a few antibacterials, such as mercury, which was used to treat syphilis, were in use when the era of true chemotherapy began. It was in the early 1900's when Paul Ehrlich first hypothesized that dyes could be used as antimicrobial drugs, based on their differential affinities for various tissues. In 1904, Ehrlich and Shiga discovered that a red dye called trypan rot was effective against trypanosomes (Mitsuhashi, 1993). It was around this time that arsenicals drew Ehrlich's interest. Ehrlich, along with Sahachiro Hata in 1909, found that arsphenamine (named Salvarsan) was active against spirochetes and, therefore, was an effective cure for syphilis (Greenwood, 2000.). The first truly effective class of antimicrobial drugs were the sulfonamides, discovered by Gerhard Domagk (Domagk, 1935). In 1932, two scientists at the Bayer company, Mietzsch and Klarer, synthesized Prontosil red, a red dye bound to sulfonamide group. Domagk (Domagk, 1935.) showed, in 1935, that infections in mice caused by hemolytic streptococci were cured by Prontosil red (Garrod, and O'Grady, 1971; Greenwood, 2000). Unfortunately for Bayer, Prontosil red was shown to have no antibacterial activity in vitro. This lack of activity was explained by Tréfouël *et al.,* 1935.) when they showed that Prontosil red is split in vivo into its component dye and sulfanilamide, the active antibacterial agent and a previously described molecule that was already in the public domain. From that point, sulfanilamide was manufactured by a number of companies and work was begun to modify the molecule to enhance performance, leading to decreased side effects and a broader spectrum of action. Although penicillin was the first natural antibiotic to be discovered, the idea of using microorganisms therapeutically was not new. Fungi had been used in poultices for many years, and by 1899, a product called pyocyanase, which was an extract from Pseudomonas aeruginosa, was used in the treatment of wounds (Garrod and O'Grady, 1971). Penicillin was first isolated from *Penicillium notatum* in 1928 by Alexander Fleming, but he was unable to isolate and purify enough drug to be of any use. By 1941, Ernst Chain, Howard Florey, and Norman Heatley had shown the therapeutic value of penicillin (Chain *et al.,* 1940.), but they were also unable to produce enough penicillin for commercial use. Collaboration with Andrew Moyer and Robert Coghill (Fleming, 1929.) at the USDA's Northern Regional Research Laboratory in Illinois led to much higher production yields of penicillin by 1943. After a worldwide search for Penicillium strains that could produce more penicillin, Raper and Fennel (Raper and

Fennell, 1946) found a strain of *Penicillium chrysogenum* on a moldy cantaloupe at a local market that was capable of even higher yields of penicillin (Demain and Elander, 1999). A series of different antibiotics were quickly discovered after penicillin came into use. In 1940, Selman Waksman began searching for antibiotic compounds produced by soil microorganisms (Greenwood, 2000). In 1943, one of Waksman's students discovered streptomycin (Schatz *et al.*, 1944), leading to a flood of researchers combing the world for new drugs. It was in this same period that Rene Dubos (Hotchkiss and Dubos, 1941) discovered gramicidin, the first antibiotic active against gram-positive bacteria. Chlortetracycline, chloramphenicol, and others were discovered shortly thereafter (Garrod and O'Grady, 1971). Many discoveries were of drugs that were too toxic for human use, or that had already been discovered. Nevertheless, this work did lead to many new drugs and within only 10 years, drugs comprising the major classes of antibiotics were found (Greenwood, 2000.). In addition to soil, many of these drugs were discovered by isolating the producing microorganisms from interesting and unusual sources. For example, some antibiotic-producing bacteria were isolated from a wound infection and others from sewage, a chicken's throat, and a wet patch of wall in Paris (Garrod and O'Grady, 1971). In 1962, one of the later discoveries was a synthetic drug, nalidixic acid, the first of the quinolones to be described, and although not therapeutically important by itself, modification of nalidixic acid led to the production of the highly effective fluoroquinolones. Members of this class, such as ciprofloxacin, norfloxacin, enrofloxacin, and ofloxacin, have become very important in the treatment of diseases in both humans and animals (Mitsuhashi, 1993.). Since the 1960's, there have been few discoveries of new antibiotic drugs. The drugs developed since have mostly been chemical modifications of existing drugs. These modifications have been very useful in treating infectious diseases, leading to enhanced killing of pathogens, increased spectrum of action, reduced toxicity, and reduced side effects. Unfortunately, since the 1970's, only one new class of antibiotics has been introduced (Lipsitch *et al.*, 2002) and a recent trend in antibiotic therapy has been to employ combinations of drugs with different mechanisms of action, in order to increase their effectiveness and to overcome the problem of drug resistance.

Tetracyclines

Tetracyclines rank among antimicrobial substances most frequently used in animal food production (Schmidt and Rodrick, 2003). Tetracyclines are broad-spectrum substances, with a wide range of activity against Gram-positive and Gram-negative bacteria, chlamydia, mycoplasma, protozoan parasites and rickettsiae (Chopra *et al.*, 1992; Roberts, 1996; Sundin, 2003). Tetracyclines according to Roberts, (1996) were the first major group of antibiotics ascribed the term "broad-spectrum". Hence, they have been used extensively in the therapy of animal and human infections, as well as for prophylactic purposes in animals and plants and for growth promotion in food animals (IOM, 1998). The chemical formula of oxytetracycline is $C_{22}H_{24}N_2O_9$ and molecular weight of 460.434 g/mol. As most tetracyclines, oxytetracycline is a polyketide with a napthecene ring, whose structure can be seen in (Figure. 1) Oxytetracycline has three pKas,.3, 7.3, and 9.1 (Rose & Pedersen, 2005), Kong *et al.*, 2012), Tamtam *et al.*, 2011),

Ahmed & Jee, 1984), a log Kow of -1.22, a log Koc ranging from 1.2-5, and a Kd ranging from 0.3 - 1030 (Chee-Sanford et al., 2009), Taylor & Chau, 1996).



Oxytetracycline (OTC)

Figure.1: Chemical structure of Oxytetracycline

Tetracyclines are continued to be used for treatment in a variety of intracellular bacteria and protozoan infections, as well as for non-infectious conditions (Chopra and Robert, 2001; Roberts, 2003). In cattle, tetracyclines are used as therapeutic agents against respiratory, urinary and local infections (Sundin, 2003). A specific indication for administering tetracyclines in cattle is infectious mastitis. A frequent and pervading source of milk contamination is intramammary (intracisternal) administration of the drug. Other milk contamination paths are percutaneous, intrauterine, subcutaneous, intramuscular and intravenous administration (Heeschen and Bluthgen, 1991). Milk tetracycline contents reach 50- 60 % concentrations of those in the blood plasma (Botsoglou and Fletouris, 2001). The overall action of tetracycline is bacteriostatic, while the main goal of the antibacterial action of the drug is protein synthesis inhibition (Navratilova *et al.,* 2009). Tetracycline binds to the bacterial 30S ribosomal subunit and prevents attachment of amino acyl tRNA to the ribosomal receptor site (Chopra *et al.,* 1992; Roberts, 1996).

Classification of tetracyclines

Tetracyclines are classified into two different types‟ i.e. typical tetracyclines and atypical tetracyclines (Michalova *et al.,* 2004).

Typical tetracyclines

A number of semisynthetic tetracyclines belong to the first class of tetracyclines referred to as "typical tetracyclines". This class exibit bacteriostatic activity by means of interacting with bacterial ribosomes and blocking of the protein synthesis (Sum *et al.,* 1998). This class include the following tetracyclines; methacycline, doxycycline, minocycline, rolitetracycline, lymecycline and glycylcyclines (Goldstein *et al.,* 1994).

Atypical tetracyclines

Atypical tetracyclines belong to the second class of tetracyclines. They include chelocardin, anhydrotetracycline, anhydro chlortetracycline and thiatetracycline. They exhibit bacteriocidal activity by targeting the cytoplasmic membrane (Oliva *et al.,* 1992; Chopra, 1994). However, due to their low-level inhibition of protein synthesis and their toxicity, these compounds are of no therapeutic interest (Michalova *et al.,* 2004).

Tetracyclines and their uses in veterinary medicine

Tetracyclines are widely used in veterinary medicine mainly for the treatment of gastrointestinal, respiratory and skin, bacterial infectious diseases of locomotive organs and of genito-urinary tract as well as systemic infections and sepsis (Prescott *et al.,* 2000). Due to their activity against a broad spectrum of pathogenic microorganisms; their absorbity, low toxicity as well as their relatively low cost, has endeared their use in the therapy of animal and human infections as well as for the prophylaxis of infections in food animals (Moellering, 1990; Standiford, 1990). World production of tetracyclines is estimated to be in thousands of tonnes annually (AHI, 2002). Consumption of tetracyclines antibiotics in veterinary practice is relatively high as compared with other classes of antibiotics (Michalova *et al.,* 2004). Sub-therapeutic levels of tetracyclines are used in certain countries as feed additives for growth promotion in animal husbandry (IOM, 1998; Schwartz *et al.*, 1998). Tetracyclines are probably the most widely used therapeutic antibiotic in food animals because of their broad-spectrum activity and cost effectiveness (Okerman *et al.*, 2004). McEroy (2002) states that tetracyclines account for more than 50 % of all in-feed antibiotics sold for use in food animals in the United Kingdom. Similarly, Van den Bogaard *et al,*. (1994) argued that the amount of tetracycline used in farm animals in the Netherlands nearly equaled that of all other antibiotics.

Tetracycline as a feed additive

Tetracyclines especially chlortetracycline and other drugs used as feed additives are essential for the purposes of growth promotion and control of diseases in dairy animals (Jenkins and Friedlander, 1982). Chlortetracycline has been used as a feed additive at concentrations ranging from 10-500g per ton of feed (Huber, 1971b). The drug is used to control calf-hood diseases in dairy cattle production (Wallace, 1970).

Mode of action of tetracyclines

Tetracyclines permeate through the bacterial cell wall by the passive diffusion and through the cytoplasmic membrane by an energy-dependent process (Franklin and Snow, 1971; Yamaguchi *et al.,* 1991; Tsankov *et al.,* 2003). Antibacterial activity of typical tetracyclines is associated with the reversible inhibition of the protein synthesis (Laskin 1976; Kersten and Frey, 1972). In addition, binding of the drug to the ribosome prevents the attachment of the amino acyl-tRNA to the site "A" of the ribosome. Tetracyclines

bind directly to the 30S-subunit protein S7 (Goldman *et al.*, 1983), other ribosomal proteins (S3, S14, and S19) are also involved (Franklin, 1966; Buck and Cooperman, 1990). Similarly, some bases in the 16S-rRNA are important for the binding of the tetracyclines to the ribosomes (Michalova *et al.*, 2004).

General aspects of antimicrobial resistance

The global emergence of antimicrobial resistance is a rising concern for human health (WHO, 2012b). The consequences of antimicrobial resistance extend beyond treatment failures in individual cases. Without effective antimicrobials, important procedures such as major surgery, organ transplantation, and cancer chemotherapy will be hazardous (Cars *et al.*, 2008; Laxminarayan *et al.*, 2013). It has been estimated that by 2050, approximately ten million deaths per year will be due to infections with resistant bacteria. This means that the number of deaths due to resistant bacteria will exceed the number of deaths due to cancer in 2050 (O'Neill, 2014). The consequences of antimicrobial resistance in animals are similar to those for humans, leading to increased suffering and mortality (Bengtsson & Greko, 2014). However, future veterinary medicine has to rely mainly on the efficacy of already existing antimicrobials (Schwarz *et al.*, 2001). Moreover, the World Health Organization has stated that some antimicrobials (fluoroquinolones, third and fourth generation cephalosporins and macrolides) should be reserved only for treating human infections (WHO, 2012a). It is also likely that any new antimicrobial will be reserved for human medicine (Schwarz *et al.*, 2001; Bengtsson & Greko, 2014). Loss of effective treatment options for animals may not only lead to therapy failures, but also to decreased welfare and reduced productivity for food-producing animals, resulting in major setbacks for the animal and global food production (Bengtsson & Greko, 2014).

Emergence and spread of antimicrobial resistance

Emergence refers to the conversion from wild-type to resistance phenotypes, whereas spread refers to the dissemination of resistance between hosts and the environment, or spread of resistance determinants between bacteria. Often emergence and spread may overlap.

Emergence

Emergence of antimicrobial resistance is a normal step in bacterial evolution, as the survival of bacteria with the phenotypical traits best adapted to the current environment (Sykes, 2010). Exposure to antimicrobials imposes a selective pressure on the bacterial population, allowing only resistant subpopulations of bacteria to survive. Antimicrobial resistance can be intrinsic or acquired (Alekshun & Levy, 2007). Intrinsic resistance is conferred by naturally occurring genes in the bacterium's genome or by inherent characteristics of the bacterium, which allow tolerance to specific antimicrobials (Alekshun & Levy, 2007; Cox & Wright, 2013). Intrinsic resistance is common for all members of a bacterial species and is independent of the selective pressure from antimicrobials (Cox & Wright, 2013). Acquired resistance is when a particular bacterium

obtains the ability to resist a specific antimicrobial agent to which it was previously susceptible (Alekshun & Levy, 2007). Unlike intrinsic resistance, acquired resistance traits are found only in some strains or subpopulations of a bacterial species (Alekshun & Levy, 2007). There are two mechanisms by which bacteria acquire resistance – by spontaneous mutations in chromosomal genes or through acquisition of naturally occurring resistance genes from other bacteria (Schwarz *et al.,* 2001; Alekshun & Levy, 2007; Sykes, 2010). Horizontal transfer of genes can occur within a bacterial species or over species boundaries either by uptake of naked DNA or through the integration of DNA in plasmids, bacteriophages, transposons, or other mobile genetic elements (Alekshun & Levy, 2007; Sykes, 2010). Many resistance genes are clustered together on mobile genetic elements, meaning that a single transfer can result in the acquisition of resistance to multiple antimicrobials (Guardabassi & Kruse, 2008). The use of antimicrobials creates optimal conditions for resistance to emerge (Guardabassi & Kruse, 2008). Exposure to antimicrobials allows AMR strains to multiply in the absence of susceptible competitors (Schwarz *et al.,* 2001). Exposure to some bactericidal antimicrobials, such as betalactams, fluoroquinolones, and aminoglycosides, may also stimulate bacteria to produce reactive oxygen species (Kohanski *et al.,* 2007). Reactive oxygen species may damage bacterial DNA, which results in the accumulation of mutations (Kohanski *et al.,* 2010). Thus, exposure to low concentrations of bactericidal antimicrobials results in formation of multidrug-resistant mutants (Kohanski *et al.,* 2010). Exposure to betalactam antimicrobials (Miller *et al.,* 2004) or reactive oxygen species (Carlsson & Carpenter, 1980) may also activate the SOS-response. The SOS-response is evoked by DNA-damage which arrests cell division and induces mutagenesis and DNA repair (Janion, 2008). This response also promotes the transfer of resistance genes by increasing the expression of genes needed for gene transfer (Beaber *et al.,* 2004). Exposure to one antimicrobial may select for resistance to other antimicrobials, because of cross- or co-resistance. Cross-resistance refers to single resistance genes or mutations conferring resistance to more than one antimicrobial class (Schwarz *et al.,* 2001; Guardabassi & Kruse, 2008). Co-resistance is the co-existence of several genes conferring resistance to different antimicrobials (Schwarz *et al.,* 2001; Guardabassi & Kruse, 2008).

Spread

Resistant bacteria or their genes do not respect ecological, phylogenetic or geographical borders and thus, the epidemiology of resistance must be seen from a holistic and global point of view (Guardabassi & Kruse, 2008). Antimicrobial resistance spreads through bacteria populations both vertically, when new generations inherit resistance determinants, and horizontally, when bacteria share or exchange resistance genes with other bacteria (Witte, 2004). Horizontal transfer of resistance genes can occur within and between bacterial species (Schwarz *et al.,* 2001; Witte, 2004). Bacteria that have acquired resistance may then spread between hosts by skin to skin contact, via excreta or saliva containing the resistant bacteria, or by exposure to contaminated food, feed, air, or water (Schwarz *et al.,* 2001). Human or animal excreta that contain resistant bacteria may contaminate the environment directly, or via the application of sludge or manure/slurry on lands (Marshall *et al.,* 2009; Wellington et al., 2013). Spread to humans and animals

then occurs through contact with soil, irrigation of crops, water, or wildlife (Wellington *et al.,* 2013). Finally, the movement of animals, food, and humans is a factor in the global dissemination of antimicrobial resistance (Laxminarayan *et al.,* 2013). When resistant bacteria have reached the new host, they can either colonize, infect, or reside only transiently (Schwarz *et al.,* 2001). In the new host, the resistant bacteria can spread their resistance genes to other bacteria, and also acquire other resistance genes from them (Schwarz *et al.,* 2001). Use of antimicrobials by some individuals may enhance the spread of resistant bacteria to other individuals sharing the same environment. First, antimicrobial treatment decreases the ratio of susceptible to resistant organisms in the bacteria population that may colonize other animals or humans (Lipsitch & Samore, 2002). Second, antimicrobial treatment reduces the competition from the residing microbiota in the treated individual, and thus, increases the treated individual's risk of being colonized with a resistant strain from the environment (Lipsitch & Samore, 2002).

Tetracycline Resistance

The prevalence of bacteria resistance to the tetracycline class of antibiotics has increased following the widespread usage of the compound within clinics, veterinary and agricultural practices (Stead *et al.,* 2007). Shortly after the discovery of tetracyclines, resistance to them was reported (Michalova *et al.,* 2004). *Shigella dysenteriae* was the first tetracycline resistant bacterium discovered and was isolated in 1953 (Watanabe, 1963; Falklow, 1975). Since then, a wide range of tetracycline resistant bacteria strains has been identified. Tetracycline resistance determinants can be found in the genomes of the physiological flora from animals, humans as well as from environmental sources and food. These bacteria can act as a reservoir of resistance genes; transfer these genes to the pathogenic genera which may lead to increasing problems of the treatment of infectious diseases (Chung *et al.,* 1999a; 1999b).The spread of bacterial resistance according to WHO (2006) has major implications. Under European Union legislation, members of the tetracycline class of antimicrobial compounds are permitted for use in the treatment of bacterial diseases in food-producing animal species. It is therefore important that effective screening and confirmatory procedures are available for detecting tetracyclines in foods of animal origin in order to provide legislators and consumers with confidence that the food products entering the food chain are compliant with the current legislation regarding permissible MRLs.

Acquired tetracycline resistance

Tetracycline resistance in most bacteria is due to the acquisition of new genes, often associated with mobile elements (Roberts, 2005). The genes are usually associated with plasmids and/or transposons and are often conjugative. Currently, there exists 38 different tetracycline resistant (tet) and oxytetracycline resistance (otr) genes described. These include 23 genes which code for energy- dependant efflux proteins, eleven (11) genes code for ribosomal protection proteins, and 3 genes which code for an inactivating enzyme and one gene with an unknown mechanism of resistance (Roberts, 2005). However, of these 38 tet genes, 8 new tet genes have been identified and the mechanism of their resistance determined (Billington *et al.,* 2002; Anderson *et al.,* 2004).

Tetracycline resistance determinants

Tetracycline resistance determinants are widespread among several bacterial species. They have been identified in 32 Gram-negative and 22 Gram-positive organisms and often found in multidrug resistant bacteria (Roberts, 1996; Levy *et al.,* 1999). According to Roberts, (1996) resistance to any drug is often due to the acquisition of new genes associated with either conjugative plasmids or transposons. Tetracycline resistance occurs by three mechanisms: the use of an energy-dependent efflux of tetracycline, altering the ribosomes to prevent effective binding of tetracycline, and producing tetracycline activating enzymes (Ng *et al.,* 2001).

Types of tetracycline resistant genes

The resistant genes associated with an efflux mechanism are tet (A), (B), (C), (D), (G), (I), (M) and (K). The tetracycline resistance genes associated with a ribosomal protection mechanism and/or efflux mechanism are tet (K), (L), (M), (O), (S), (P), (Q), (B), (D), (H) and (C). While tet (X) is the only example of tetracycline resistance gene causing the enzymatic alteration of tetracycline (Ng *et al.,* 2001). Thirty classes of tetracycline resistance have been identified based on DNA-DNA hybridization with regions from structural genes and DNA sequencing (Tenover *et al.,* 1987; Scott and Rood, 1989; Zhao and Aoki, 1992; Leng *et al.,* 1997).

Identification of new tet genes

A number of new genera have been identified carrying previously described tet (A), tet (B), tet (C), tet (D), tet (G), tet (H), tet (K), or tet (L) efflux genes and/or tet (M), tet (O), tet (S), tet (Q), or tet (W) ribosomal protection genes (Chopra and Roberts, 2001; Chung *et al.,* 2002). The current information reflects the examination of tetracycline resistance (Tcr) bacteria from a variety of ecosystems, new species and genera, as well as the continued spread of tet over time (Kim *et al.,* 2004). Furthermore, new conjugative transposons, carrying different ribosomal protection tet genes, have been identified, and many are related to the Tn 916-Tn 1545 family of elements (Brenciani *et al.,* 2004; Lancaster *et al.,* 2004;). There are reports on an increase in the percentage of Gram-negative isolates which carry multiple tet genes (Wolkerson *et al.,* 2004).

Mechanism of tetracycline resistance

Three different mechanisms of tetracycline resistance have been described (Franklin and Snow, 1971; Burdett, 1986; Speer *et al.,* 1991). All the mechanisms are based on the acquisition of one or several tetracycline resistance determinants, which are widely distributed among bacterial genera (Schnappinger and Hillen, 1996). Additionally, mutations in the rRNA, multidrug transporter systems or permeability barriers may be involved in the resistance to several antibiotics including tetracyclines (Michalova *et al.,* 2004). Furthermore, thirty three different tetracycline resistance (tet) genes and three oxytetracycline resistance (otr) genes have been described (Roberts, 2003). Moreover,

there is no essential difference between the tet and otr genes, but oxytetracycline resistance genes were first described in oxytetracycline producing animals (Ohnuki *et al.,* 1985; Doyle *et al.,* 1991; Levy *et al.,* 1999).

Active efflux proteins

Twenty four (60 %) of all tet genes code for energy-dependent membrane associated proteins which exports tetracycline out of the cell (Roberts, 2005). This action reduces the intercellular concentration of tetracycline and protects the bacterial ribosomes in vivo. The efflux proteins exchange a proton for a tetracycline-cation complex against a concentration gradient. These genes are the most commonly found tet genes in Gram-negative bacteria (Chopra and Roberts, 2001). Efflux of tetracycline is mediated by energy-dependent efflux pumps. Efflux proteins, located in the cytoplasmic membrane exchange a proton for a mono cationic magnesium-tetracycline complex. They work as antiporters and thus reduce the amount of the antibiotic in the cytoplasm (Sum *et al.,* 1998). The regulation of tet gene expression differs in Gram-positive and Gram-negative bacteria. In Gram-negative bacteria, each determinant consists of two genes coding for an efflux protein and a repressor protein, both regulated by tetracycline (Michalova *et al.,* 2004). They originated divergently and share the central regulatory region. In the absence of tetracycline, the repressor protein TetR binds to the operator of the structural efflux gene and thus blocks its transcription (Hillen and Berens, 1994). Induction occurs when the Mg2+-tetracycline complex formed in the cell binds to the repressor, and conformation changes of the repressor lead to its release from the operator allowing the transcription of the structural efflux gene. The repressor binds again to the operator if the intracellular amount of tetracycline decreases (Roberts, 1996; Michalova *et al.,* 2004).

Ribosomal protective proteins

Ribosomal protection was first discovered in *streptococci*, and is the second most important mechanism of tetracycline resistance in bacteria after the active efflux (Burdett, 1986). Ribosomal protective proteins ensure the resistance to tetracycline, doxycycline as well as minocycline (Sanchez-Pescador *et al.,* 1988; Taylor and Chau, 1996). Ribosomal protective proteins might confer resistance by means of the reversible binding to the ribosome (Schnappinger and Hillen, 1996). There are eleven tet genes coding for ribosomal protective proteins (Saphn *et al.,* 2001; Cornell *et al.,* 2003). Under normal conditions, the ribosomes are in standard configuration and function normally. This balance is changed with the introduction of tetracycline into the system (Cornell *et al.,* 2003). The tetracycline binds to the ribosome's configurational state which disrupts the elongation cycle and protein synthesis stops. The ribosomal protection proteins are believe to interact with the base of h34 protein, within the ribosome, causing an allosteric disruption of the primary tetracycline binding site(s) and the tetracycline molecules are released from the ribosomes (Roberts, 2005). The ribosome returns to its standard conformational state and protein synthesis proceeds. Whether the ribosomal proteins actively prevent tetracycline from binding to the ribosomes after they have been released is not known, nor is it known if once the tetracycline is released whether it can rebind to the same or a different ribosome and inhibit protein synthesis again (Saphn *et al.,* 2001).

Enzymatic inactivation

The gene tet (X) is the only example of tetracycline resistance due to the enzymatic modification and inactivation of the antibiotic (Speer *et al.,* 1991). This gene has been discovered on two Bacteroides transposons, Tn 4531 and Tn 4400, and has been found to share considerable amino acid homology with a number of NADPH-requiring oxidoreductases (Michalova *et al.,* 2004). The tet (X) gene encodes for an NADPH-requiring oxidoreductase, which inactivates tetracycline in the presence of oxygen and NADPH, but has only been found in a strict anaerobe, where oxygen is excluded (Chopra and Roberts, 2001). The tet (X) gene has a percentage G+C content of 37.4 % suggesting that it is of Gram-positive ancestry and is active in aerobic E. coli (Diaz-Torres *et al.,* 2003).

Multidrug-resistance mechanisms

In addition to the specific mechanisms of tetracycline resistance encoded by tetracycline resistance genes, other mechanisms such as the multidrug-resistance can contribute more or less to the resistance to tetracyclines in certain bacteria genera (Michalova *et al.,* 2004). These common mechanisms include mutations, permeability barriers or multidrug transporter systems. Mutations The discovery of a mutation in the 16S-rRNA that conferred the resistance to tetracyclines in Gram-positive was reported by Ross *et al.,* (1998). This mutation consists in the change of a single base (G-C) at the position cognate with E. coli 16S-rRNA base 1058 (Moine and Dahlberg, 1994). Another mutation in the 16S-rRNA was revealed showing a high-level resistance to tetracycline in *Helicobacter pylori*. Identical triple base-pair substitution located in the primary binding sites of tetracycline was discovered by several studies (Geritts *et al.,* 2002; Trieber and Taylor, 2002). However, these substitutions (single and double) mediated only low-levels of tetracycline resistance (Dailidiene *et al.,* 2002).

Permeability barriers

Outer membrane of Gram-negative barrier represents the first effective barrier to the various compounds and this plays a role in the antimicrobial resistance. Porins, the major outer membrane proteins, form channels in the outer membrane and allow the nonspecific passages of small polar molecules, amino acids or nutrients (Nikaido, 1994). The rapid passage of tetracyclines into the cell occurs preferentially via the outer membrane protein F (ompF) and in the magnesium-bound form of tetracycline. Whereas, in the porin-defficient cells the influx of the drug is slow, mainly in its unchanged form (Thanassi *et al.,* 1995). Therefore, the decreased level of ompF synthesis leads to the increased level of tetracycline resistance (Cohen *et al.,* 1989). In addition to the decreased number of porin channels in the outer membrane, several studies have revealed mutations and amino acid changes that influence the structure and the function of porin (De *et al.,* 2001; Olesky *et al.,* 2002). Multidrug transporters Multidrug transporters play important role in tetracycline resistance almost in Gram-negative bacteria (Michalova *et al.,* 2004). On the basis of the energetic criteria, they can be divided into two classes separating multidrug

transporters utilizing a proton motive force (PMF) for the exudation of drugs from the cell, and ATB binding cassette (ABC) multidrug transporters that gain the energy for the efflux from the ATP hydrolysis (Paulsen *et al.,* 1996a; Putman *et al.,* 2000). Within the class of PMF transporters, distinct families of proteins have been distinguished: the major facilitator superfamily (MFS), the small multidrug resistance (SMR) family, the resistance nodulation-cell division (RND) family and the multidrug and toxic compound extrusion (MATE) family (Marger and Saier, 1993; Saier *et al.,* 1994; Paulsen *et al.,* 1996b; Brown *et al.,* 1999). The EmrE multidrug transporter (also called Mvrc) of *E. coli* was originally identified on the basis of its ability to confer resistance to ethidium bromide and methyl violgen (Purewall, 1991; Morimyo *et al.,* 1992). The EmrE is a member of SMR family which unifies small efflux proteins that function as drug or proton antiporters and export drugs to the periplasmic space (Nikaido, 1998). Over production of EmrE protein results in the low-level resistance to tetracycline and several other antibiotics (Ma *et al.,* 1994).

Linkages of tet genes with mobile elements

The tet genes are often associated with plasmids, transposons and conjugative transposons which may carry other antibiotic resistance and/or heavy metal resistance genes (Chopra and Roberts, 2001). Many of these elements code for their own transfer, and may greatly influence their ability to spread to new genera (Roberts, 2005). Integrons have been identified in Gram-negative genera (Chopra and Roberts, 2001), but tet genes have not yet been found within integrons, which function as a general gene-capture system, and allow multiple antibiotic genes to be linked (Recchia and Hall, 1995). A new generation of tetracycline, the glycylcyclines (tigecycline) have been developed to overcome bacterial resistance due to tet genes coding for efflux proteins or ribosomal protection proteins (Chopra, 2002; Zhanel *et al.,* 2004). No tigecycline resistant bacteria have been identified in nature, however, it is possible that bacteria carrying acquired tet genes may have their tet genes mutated and become more resistant to this antibiotic. Therefore, it is unclear how this antibiotic will impact bacterial acquisition and spread of acquired tet genes. It is unlikely that overall use of tetracyclines will change in the near future, especially in countries where tetracyclines are used as growth promoters. Thus the trends will most likely show continued increase in the number of tetracycline resistant genera and the percent of bacterial population no longer susceptible to tetracyclines (Roberts, 2005).

Methods of determining tetracycline resistance in microorganisms

Antimicrobial susceptibility testing for tetracycline resistance can be accurately performed by either dilution or disk diffusion methods and by genetic methods (Jorgensen *et al.,* 1999).

Dilution method

Dilution tests results in quantitative minimal inhibitory concentration (MIC) value in microorganisms per milliliter. The MIC is the lowest concentration of an antimicrobial

agent that prevents visible growth of a microorganism in an agar or broth dilution susceptibility test (NCCLS, 2002a). This method is based on the inoculation and growth of the microorganism in media containing different concentrations of an antimicrobial agent. This procedure is done by the agar-based or the broth-based method and the concentration range used depends on the antimicrobial drug and on the microorganism tested (Michalova et al., 2004). The result obtained is reported as quantitative MIC value in μg/ml and/or as the classification of the microorganism into categories: susceptible, intermediate or resistant, based on the interpretive standards (Jorgensen *et al.,* 1999; NCCLS, 2002b). According to the MIC interpretive standards, recommended by NCCLS (2000a; 2000b), microorganisms other than streptococci are considered to be resistant to tetracycline if MIC ≥ 16 μg/ml, intermediate if MIC = 8 μg/ml and susceptible at MIC at ≤ 4 μg/ml. While *streptococci* are considered resistant if MIC ≥ 8 μg/ml, intermediate at MIC = 4 μg/ml, while strains are considered susceptible at MIC values ≤ 2 μg/ml.

Disk diffusion method

This test results in qualitative information about the susceptibility of the microorganism (Schlegelova and Rysanek, 1999). Commercially prepared paper disks impregnated with a defined amount of antibacterial agent are used for this test. The amount for each antimicrobial agent in the disk is standardized (NCCLS, 2000b). The method depends on the diffusion of the drug from the disk and the creation of the concentration gradient in the agar medium surrounding the disk. Disks are applied onto the surface of the agar medium inoculated with a microorganism and after the incubation, the diameter of the zone with suppressed growth is measured (Bauer et al., 1966; NCCLS, 2000b). The recommended interpretive standards by NCCLS (2000ab) include: microorganisms other than *streptococci* are considered resistant to tetracycline if the diameter of the zone of growth of inhibition is ≤ 14 mm, 15 and 18 mm as intermediate and ≥ 19 mm as susceptible when using a disk impregnated with 30 μg of tetracycline. While for *streptococci* a zone diameters of ≤18, 19 to 22 and ≥ 23 are considered resistant, intermediate and susceptible respectively.

Genetic methods for the detection of antimicrobial resistance genes

Genetic methods may confirm the presence of specific genes conferring tetracycline resistance. However, the presence of genes alone does not necessarily mean resistance of the microorganism, as it is possible (although unlikely) that resistance genes may not be expressed. Genetic methods can be fast and it is possible to use them directly on clinical specimens (Tenover and Rasheed, 1999). The most used methods is PCR (polymerase chain reaction) with specific primers for specific resistance genes; although DNA hybridization, using specific labeled molecular probes is another method for detecting resistance genes. Multiplex PCR using several pairs of primers for several different resistance genes in a single reaction may allow the detection of more than one resistance genes at a time (Warsa *et al.,* 1996; Ng *et al.,* 2001).

The Bacterium *Escherichia coli*

Escherichia coli historically was first isolated from the faeces of a child in 1885 by Theodor Eschrich and have since remained the most studied bacterium. It is a common inhabitant of the gastrointestinal tract of animals and humans (Kaper *et al.,* 2004). There are two broad types of *E. coli*, the *E. coli* strains that are harmless commensals of the intestinal tract and others that are major pathogens of human and animals. While the pathogenic *E. coli* strain is divided into those causing disease inside the intestinal tract and others capable of infection at extra-intestinal sites (Sousa, 2006). *Escherichia coli* is found secondarily in soil and water due to faecal contamination. The bacterium can be cultured easily in the laboratory, the different pathogenic genotypes can be identified through virulence gene detection methods (Sousa, 2006). Coliform bacteria possess variation in their morphology including E. coli. The usual morphology observed in the stained preparation after culture on nutrient agar range from 2 to 4 microns in length and 0.4 to 0.7 microns in breadth (Hahn, 1996). Escherichia coli frequently contaminate food and can serve as a good indicator of faecal pollution (Dilielo, 1982; Soomro *et al.,* 2002; Benkemoun *et al.,* 2004). *Escherichia coli* is the most prevalent infecting organism in the family of gram-negative bacteria known as *enterobacteriaceae* (Eisenstein and Zaleznik, 2000). *E. coli* that are responsible for the numerous reports of contaminated foods and beverages are those that produce Shiga toxin, so called because the toxin is virtually identical to that produced by *Shigella dysenteria* type 1 (Griffin and Tauxe, 1991). The best-known and also most notorious *E. coli* bacteria that produce Shiga toxin is *E. coli* O157:H7 (Griffin and Tauxe,1991; Eisenstein and Zaleznik, 2000). Shigatoxin producing *E. coli* (STEC) causes approximately 100,000 illnesses, 3,000 hospitalizations and 90 deaths annually in the United States (Mead *et al.,* 1999). Most reported STEC infections in the United States are caused by *E. coli* O157:H7, with an estimated 73,000 cases occurring each year (Mead *et al.,* 1999). *Escherichia coli* is one of the main inhabitants of the intestinal tract of most mammalian species, including humans, cattle and birds. Shiga toxin-producing *E. coli* (STEC), also called verotoxinogenic *E. coli*, do not cause disease in animals but may cause watery diarrhoea, haemorrhagic colitis, and/or haemolytic uraemic syndrome in humans (Fairbrother and Nadeau, 2006). Zoonotic STEC include the O157:H7 strains and, with increasing frequency, certain non-O157 strains. The importance of non-O157 zoonotic strains is probably underestimated as they have been less well characterized and are more difficult to detect in samples than O157:H7 (Fairbrother and Nadeau, 2006). Another large subset of STEC strains has been isolated from animals but has not, at the present time, been associated with disease in animals or humans. Cattle and other ruminants are the most important reservoir of zoonotic STEC, which are transmitted to humans through the ingestion of foods or water contaminated with animal faeces, or through direct contact with the infected animals or their environment (Fairbrother and Nadeau, 2006). Pathogenic members of the coliform group as well as the *Enterobacteriacae* family are represented by genera such as *Salmonella* and *Shigella* and, are often found in the intestines of humans and animals (Le Minor, 1984; Rowe and Gross, 1984; Collins *et al.,* 1995; Hayes *et al.,* 2001). Most strains of *E. coli* are non-pathogenic (Stender et al., 2001). However some strains differ from commensal in that they express virulence factors directly involved in pathogenesis thereby causing disease (Schroeder *et al.,* 2004). E. coli frequently contaminates food and is a good indicator of faecal pollution (Dilielo, 1982; Soomro *et al.,* 2002; Benkemoun *et al.,* 2004). Presence of pathogenic *E. coli* in milk

products indicates the presence of enteropathogenic microorganisms, which constitute a public health hazard.

NTSEC is chosen as the indicator commensal organism because ti is easy to isolate from all animals and is one of the major carcass contaminants at slaughter (Stopforth *et al.,* 2006). It is representative of Gram-negative bacteria. Monitering AMR of this commensal gives a measure of selection pressure on the microflora in that animal and allows comparison and contrast of AMR from different species (McEwen *et al.,* 2006a). NTSEC is considered a potential reservoir of AMR genes that could transfer AMR to other zoonotic or commensal organisms that might cause disease in cattle or people (Blacke *et al.,* 2003; Hart *et al.,* 2006; Linton *et al.,* 1977b; Winokur *et al.,* 2001).

Materials and Methods

Sample collection and preliminary bacterial culture

In this study, a total of 53 different clinical samples were collected from cow, sheep, goat, dog and cats, which presented to Al Muthanna veterinary hospital from October to December 2017. The samples were collected aseptically using sterile swab after disinfected the area of collection with 70% alcohol to minimize surface contamination. All samples were transferred in cool box to the clinical pathology laboratory/ College of Veterinary Medicine/ Al Muthanna University.
All samples were culture on 5% sheep blood and MacConkey agar (Figures 2) (Merck, Germany) and incubated for 18 to 24 h at 37 °C. All bacterial isolates were re-cultured on differential media and nutrient agar for further identification procedures. Colonies with the typical color and appearance of *E. coli* were picked and streaked again on blood agar plates and re-streaked on EMB agar (Merck, Germany). Greenish metallic sheen growth was suggestive of *E. coli* which was inoculated on nutrient slants for further biochemical tests. The *E. coli* isolates were stored in tryptic soy broth (Merck, Germany) with 15% glycerol at –20 °C (Mooljuntee *et al.,* 2010).

Figure. 2: shows the preparation of media

Conventional biochemical test

All Gram positive and negative bacterial isolates including *E.coli* suspected colonies on EMB were further screened by means of biochemical tests namely; Simmon citrate, Urea, Tripple Iron Sugar (TSI), sulfate, Indole, Motility (SIM), Methyl Red (MR), Vogesproskeur (VP), oxidase and catalase. Various reactions of the tests such as color change, motility and gas formation were used to interpret results as either positive or negative after 24 hour incubation. These tests were carried out as described in the methodology of Khandaghi *et al.,* (2010) (Figure. 3 & 4).

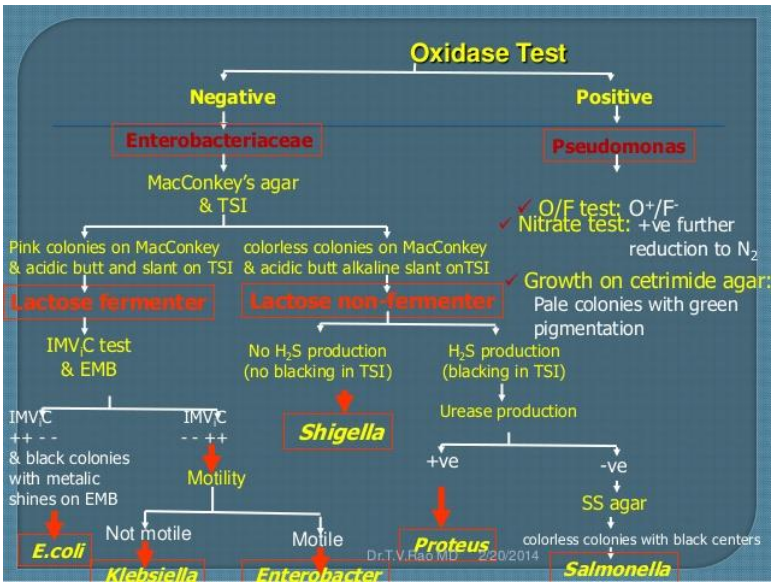


Figure.3: Shows the steps in the identification of Gram positive and negative bacteria

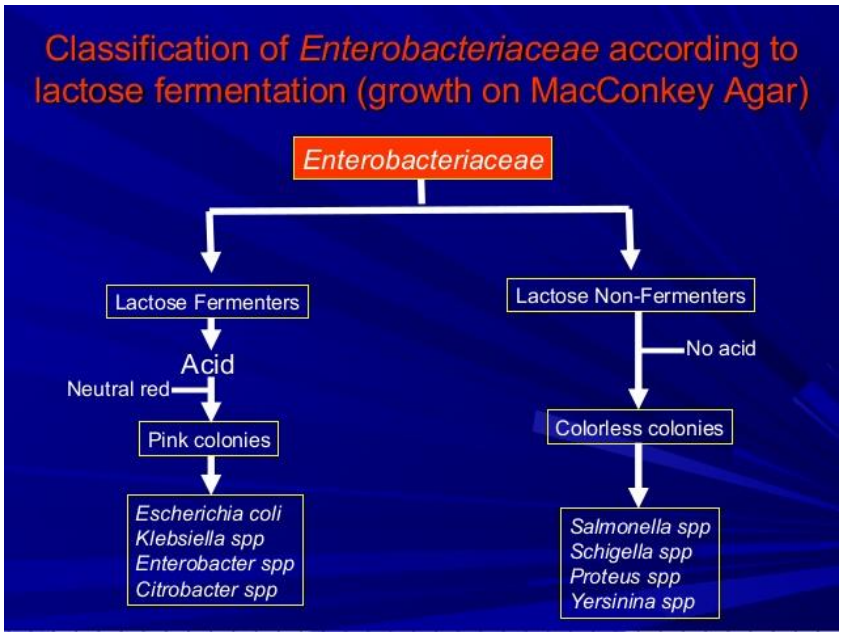


Figure.4: Shows the steps for the identification of *E. coli*

Triple sugar iron agar test (TSI)

In this test, the Triple Sugar Iron Agar was prepared according to the manufacturer‟s instruction TSI tube was inoculated with the isolates both on the butt and the slant by stabbing and streaking respectively. This was followed by incubation at 37°C for 24-48 hours. It was then observed for hydrogen sulfide production (which is indicated by a black precipitate at the butt of the tube) and carbohydrate fermentation (indicated by gas production and colour change), (Carter, 1986) (Figure. 5).

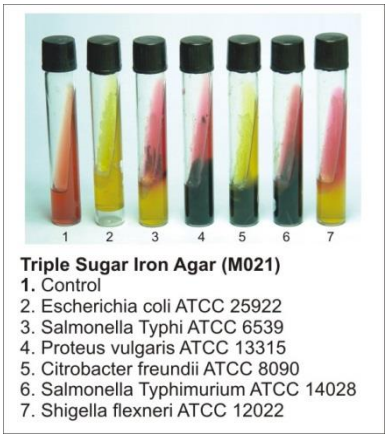


Figure. 5: Shows the stander reading of TSI

Sulphur, indole and motility tests (SIM)

The Sulphide, Indole Motility, SIM media was prepared according to the manufacturer‟s instruction. The pure isolates were inoculated into the medium by stabbing and incubated at 37oC for 18-24hrs. They were then observed for hydrogen sulphide (H2S) production, (indicated as a black coloration in the tube) and motility (indicated by migratory movement along the line of stabbing). Three drops of Kovac‟s indole reagent were then added and shaken gently. After one minute, a positive reaction was indicated by the development of a red color in the reagent layer above the medium which is indicative of *Escherichia coli*.

Methyl Red-Voges Proskauer test

Pure isolates of the non-sorbitol fermenters were inoculated into 5ml of MR-VP broth and incubated for 48hrs at 35 ^{0}C. After incubation, about 1ml of the broth was transferred to a small serological tube followed by the addition of 2-3drops of methyl red and the color on the surface of the medium was read immediately. A red coloration on addition of the indicator signified a positive methyl red test. To the rest of the broth in the original tube, 5drops of 40% potassium hydroxide (KOH) were added followed by 5drops of 5% of alcoholic (ethanol) alpha-Naphthol and shaken. The cap of the tube was loosened and placed in a sloping position. The development of a red color starting from the liquid-air interface within 1hour indicates a positive test. Escherichia coli are reported to be Methyl red positive with an orange to red coloration and Voges-Proskauer negative with no coloration (Cheesbrough, 1985).

Citrate utilization test

In this test a sterile needle was used to pick a single isolated colony which were lightly streaked on the surface of the Simmon‟s citrate agar slant (Prepared according to manufacturer‟s instruction), which contains a pH indicator (Bromothymol blue) in a test tube (whose screw cap was placed loosely) and incubated at 350C for 18-24hours. At neutral pH as a result of organism present not utilizing citrate, a green coloration of the indicator was observed, thus indicating a negative test. Escherichia coli are reported to be distinctively citrate negative (MacFaddin, 2000; Reddy, 2007).

Urease test

In this test, pure culture was used to streak the entire surface of the urea agar slant prepared in a test tube under sterile conditions. The inoculated test tubes were then incubated for 18-24 hours at 37oC. Urease production is indicated by a bright pink (fuchsia) color on the slant which identifies those organisms that are capable of hydrolyzing urea to produce ammonia and carbon dioxide, of which *E. coli* is negative for, indicated by the culture medium remaining yellowish in color. (MacFaddin, 2000).

Determination of Antibiotic Susceptibility of *E. coli* Isolates

Antibiotic susceptibility profile of each isolate was determined using the disc diffusion method according to the CLSI protocol (2006a). Colonies (4-5) of the test isolates from overnight cultures on EMB plates were picked and emulsified in sterile normal saline. The turbidity of the suspension was adjusted to match 0.5 MacFarland‟s standard. Ten µl of the suspension was then dispensed and spread on Mueller-Hinton agar plates to create a uniform lawn. The pre-inoculated plates were used for the disc diffusion test. The isolates were tested with a panel of **6 antibiotic** discs (tetracycline (T30), and oxytetracycline (TE10), streptomycin (S25), penicillin (P10), trimethoprim-sulphamethoxazoles (SXT25) and Chloramphenicol (C10) discs. the antibiotic discs were placed on the surface of each of the pre-inoculated Mueller-Hinton plates using a disc dispenser (Oxoid UK) and the plates incubated aerobically at 37 ^{0}C for 24 hours. After the incubation period the diameters of the antibiotic inhibition zones were measured to the nearest millimeter (mm) using a digital meter ruler (Figure. 4) and were classified as susceptible (S), intermediate resistant (I) or resistant (R) according to the CLSI (2006b) criteria. Antibiotic discs were obtained from the Oxoid Company (UK) (Figure.5).

Figure. 5: Shows the digital meter ruler that used for measure the inhibition zones

Detection of Tetracycline (tet) Resistance Genes

Isolation of genomic DNA from *E. coli* bacterial broth cultures

The DNA was extracted and purified according to the instructions of the company (Qiagen/German).

Procedure

1- Bacteria was cultured overnight.
2- Pipet 1 ml of bacterial culture into a 1.5 ml microcentrifuge tube, and centrifuge for 5 min at 5000 x g (7500 rpm). 3-Calculate the volume of the pellet or concentrate and add Buffer ATL (supplied in the QIAamp DNA Mini Kit) to a total volume of 180 µl. 4- Add 20 µl proteinase K, mix by vortex, and incubate at 56°C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample, or place in a shaking water bath.
5- Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.
6- Add 200 µl Buffer AL to the sample, mix by pulse-vortex for 15 s, and incubate at 70°C for 10 min. briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid. 7-Add 200 µl ethanol (96–100%) to the sample, and mix by pulse-vortex for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid

8-Carefully apply the mixture from step 6 (including the precipitate) to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.
9-Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.
10- Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min.
11-Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min. This step helps to eliminate the chance of possible Buffer AW2 carryover.
12- Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 µl Buffer AE or distilled water. Incubate at room temperature for 1 min, and then centrifuge at 6000 x *g* (8000 rpm) for 1 min.
13- Repeat step 11.

Table. 1: Shows the designed primers use for confirmation of the tet A resistance gene

Prime	Sequence (5'-3')	Size of amplified product (bp)	References
*tet*AC F	5'CGCYTATATYGCCGAYATCAC-3'	417bp	**(Balasubramaniam, et al., 2003)**
tetAC R	5'CCRAAWKCGGCWAGCGA-3		
Tet(A)-F	5'- GTGAAACCCAACATACCCC-3'	888bp	**(Maynard *et al.*, 2003)**
Tet(A)-R	5'-GAAGGCAAGCAGGATGTAG-3		

Table. 2: PCR Amplification cocktail Tet(A)

Reagents		Quantity
1.	Green Master Mix. Tube	10 µl
2.	F Primer	1 µl
3.	R Primer	1 µl
4.	DNA template	6 µl
5.	Nuclease-free water	32 µl

Table. 3: PCR Amplification Program Tet(A)

Stages		Steps	Temperature (C˚)	Time	No. of cycles
First		Initial Denaturation	94	5 min	1
Second	I	Denaturation	94	40 s	40
	II	Annealing	57	1	
	III	Extension	72	1 min	
Third		Final Extension	72	10 min	1

Table. 4: PCR Amplification Program tetAC

Stages		Steps	Temperature (˚ C)	Time	No. of cycles
First		Initial Denaturation	94	5 min	1
Second	I	Denaturation	94	45 s	30
	II	Annealing	55	1 min	
	III	Extension	72	45s	
Third		Final Extension	72	10 min	1

Results

Microbiological observations

In this study, totally 53samples including 9, 37,6 and 1 were collected from cow , sheep, goat and cat respectively. The total number of isolated bacteria were 53 isolates (Table. 5).

Table. 5: Shows the total number and bacterial isolates from animals

Species	**Number of animals**	**No of isolates**
Cow	9	9
sheep	37	37
goat	6	6
cat	1	1
Total	53	53

Fifty three bacterial isolates were isolated from different animal specimens. All isolates revealed were revealed typical Gram staining, colonies morphological appearance and biochemical tests (Figure. 6). Ten selected *E. coli* isolates were identified by produced characteristic reaction in biochemical tests (Table-6).

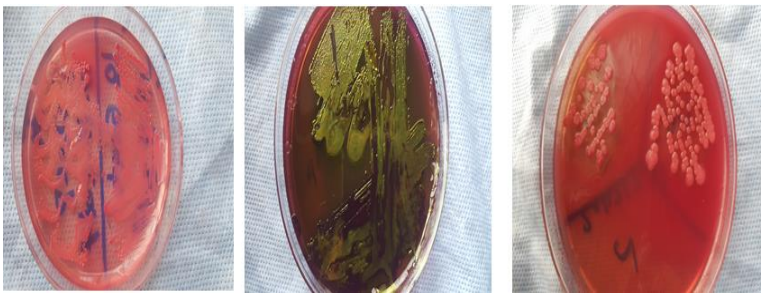


Figure.6: Shows the different bacterial isolates on culture media

Table.6: Results of biochemical tests used for identification of *Escherichia coli.*

Gram	-negative, small rod
Pink colonies	MacConkey agar
Colonies with green metallic sheen	eosin-methylene blue agar
Negative	citrate test
Negative	oxidase test
Positive	indole test
Positive	methyl red test
Negative	Voges-Proskauer test
Positive	catalase production
Positive	lactose fermentation
Positive	urea hydrolysis
Positive	nitrate Reduction
Positive	gelatin hydrolysis

Positive for case in hydrolysis	

In samples collected from sheep, the number of bacterial isolates were 25, 6, 3, 1 and 1 for *E. coli*, *Mannheimia haemolytica, Pasteurella Multocida, Klebsiella, Proteus* and *Samonella* respectively. The result of this study also revealed that *E. coli* was the most common isolated bacteria. The majorities of these isolates were resistance to the panel of antibiotic discs that used in this study. The percentages of resistance were 81.57%, 92.1%, 84.21%,89.47%,94.7%, 84.21% for S, Te, C, T, P and SXT respectively. (Table.7).

Table.7 : Shows the bacterial isolates from samples that collected from sheep and the resistances of bacteria to antibiotic discs.

Name of MO	**Isolated number**	**SXT**	**P**	**T**	**C**	**Te**	**S**
E.coli	25	20	24	23	21	24	20
Mannheimia haemolytica	6	6	6	6	6	6	6
Pasteurella Multocida	3	2	3	2	2	2	2
Klebsiella	2	2	2	2	2	2	2
Proteus	1	1	0	0	0	0	0
Salmonella	1	1	1	1	1	1	1
Total	38	32	36	34	32	35	31
Percentage		84.21%	94.7%	89.47%	84.21%	92.1%	81.57%

In samples collected from goat, the number of bacterial isolates were 4,1 and 1 for *E.coli Stapylococcus and Micococcus* respectively. The result of this study also revealed that *E. coli* was the most common isolated bacteria. The majorities of these isolates were resistance to the panel of antibiotic discs that used in this study. The percentages of resistance were 100% 100%, 83.33%, 100%, 83.33% and 100% for S, Te, C, T, P and SXT respectively. (Table.8).

Table.8 : Shows the bacterial isolates from samples that collected from sheep and the resistances of bacteria to antibiotic discs.

Name of MO	**Isolated number**	**SXT**	**P**	**T**	**C**	**Te**	**S**
E.coli	4	4	4	4	4	4	4
Stapylococcus	1	1	1	1	1	1	1
Micococcus	1	1	0	1	0	1	1
Total	6	6	5	6	5	6	6
Percentage		100	83.33%	100%	83.33%	100%	100%

In samples collected from cow, the number of bacterial isolates were 4, 1,1, 1 and 2 for *E.coli, klebsiella, pasteurella, Streptococcus and Proteus* respectively. The result of this study also revealed that E. coli was the most common isolated bacteria. The majorities of these isolates were resistance to the panel of antibiotic discs that used in this study. The percentages of resistance were 77.77%, 100%, 77.77%,100%,100% and 77.77% for S, Te, C, T, P and SXT respectively. (Table.9).

Table.9 : Shows the bacterial isolates from samples that collected from sheep and the resistances of bacteria to antibiotic discs.

Name of MO	Isolated number	SXT	P	T	C	Te	S
E.coli	4	2	4	4	2	4	2
klebsiella	1	1	1	1	1	1	1
pasteurella	1	1	1	1	1	1	1
Streptococcus	1	1	1	1	1	1	1
Proteus	2	2	2	2	2	2	2
Total	9	7	9	9	7	9	7
Percentage		77.77%	100%	100%	77.77%	100%	77.77%

Only one *E.coli* isolate was isolated from fecal samples of cat with different reaction to antibiotic discs panel, which was R, 11.52, R, 5.5, 12.1, 13.8 and 1 for S, Te, C, T, P and SXT respectively (Figure. 6).

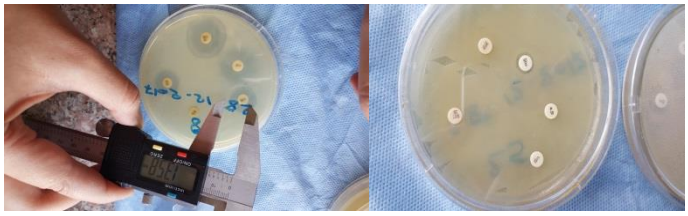


Figure.7: shows the antibiotic sensitivity test.

All data of bacterial isolates and the number of resistance bacteria and its percentages are presented in the Table. 10, 11and 12 for cow, sheep and goat respectively

Table.10: Shows details of isolated bacteria from different samples of cow (FS= Fecal sample, RF=Ruminal fluid, NS= Nasal swab, S= streptomycine, Te=Oxytetracycline, C= chloramphenicol, T= Tetracycline , P= Pencilline, SXT, Sulfa-Trimethoprim)) .

S	Te	C	T	P	SXT	Bacteria	Sample
R	R	R	R	R	R	E.coli	FS
10.78	R	12.3	R	R	7.55	E.coli	RF
R	R	R	R	R	R	klebsiella	RF
R	R	R	R	R	2	E.coli	FS
5	R	6.2	R	R	R	E.coli	FS
R	R	R	R	R	R	pasteurella	NS
R	R	R	R	R	R	Streptococcus+ Proteus	Pus Hernia
R	R	R	R	R	R	Proteus	Pus Knee joint

Table.11: Shows details of isolated bacteria from different samples of sheep ((FS= Fecal sample, RF=Ruminal fluid, NS= Nasal swab, S= streptomycine, Te=Oxytetracycline, C= chloramphenicol, T= Tetracycline , P= Pencilline, SXT, Sulfa-Trimethoprim) .

S	**Te**	**C**	**T**	**P**	**SXT**	**Bacteria**	**Sample**
R	R	14.41	R	R	R	E.coli	FS
R	R	R	R	R	R	E.coli	FS
R	R	R	R	R	R	E.coli	FS
8.75	R	10.85	R	R	12.16	E.coli	FS
3.91	R	R	5.3	R	8.31	E.coli	FS
R	R	R	R	R	R	E.coli	FS
7.74	R	18.7	R	R	R	E.coli	FS
R	R	R	R	R	R	E.coli	FS
R	13.8	R	15.1	R	14.5	E.coli	FS
R	R	R	R	R	R	E.coli	FS
R	R	R	R	R	R	E.coli	FS
R	R	R	R	R	R	E.coli	FS
11.77	R	R	R	R	20.4	E.coli	FS
R	R	R	R	R	R	E.coli	FS
R	R	R	R	R	R	E.coli	FS
R	R	R	R	R	R	E.coli	FS
R	R	R	R	R	R	E.coli	FS
7.74	R	R	R	R	R	E.coli	FS
R	R	R	R	R	R	E.coli	FS
R	R	R	R	R	R	Salmonella	FS
R	R	10.28	R	R	R	E.coli	FS
R	R	R	R	R	R	klebsiella	FS
7.65	13.9	10.5	16.53	5	R	Proteus	FS
10.2	7.79	12.27	11.65	R	16.78	Pasteurella	NS
R	R	R	R	13.7	20.54	E.coli	NS
R	R	R	R	R	R	Mannheimia haemolytica	NS
R	R	R	R	R	R	Mannheimia haemolytica	NS
R	R	R	R	R	R	Mannheimia haemolytica	NS
R	R	R	R	R	R	Pasteurella multocida	NS
R	R	R	R	R	R	klebsiella	NS
R	R	R	R	R	R	E.coli	NS
R	R	R	R	R	R	E.coli	NS
R	R	R	R	R	R	Pasteurella multocida	NS
R	R	R	R	R	R	Mannheimia haemolytica	NS
R	R	R	R	R	R	Mannheimia haemolytica	NS
R	R	R	R	R	R	Mannheimia haemolytica	Lung
R	R	R	R	R	R	E.coli	Eye swab

Table.12: Shows details of isolated bacteria from different samples of sheep ((FS= Fecal sample, RF=Ruminal fluid, NS= Nasal swab, S= streptomycine, Te=Oxytetracycline, C= chloramphenicol, T= Tetracycline , P= Pencilline, SXT, Sulfa-Trimethoprim)

S	Te	C	T	P	SXT	Bacteria	Sample
R	R	R	R	R	R	E.coli / hemolysis	FS
R	R	R	R	R	R	E.coli / hemolysis	FS
R	R	R	R	R	R	E.coli	FS
R	R	R	R	R	R	E.coli	FS
R	R	R	R	R	R	Staphylococcus	Ear pus
R	R	7.4	R	12.2	R	Micrococcus	Milk sample

Results of Tetracycline (tet) Resistance Genes

Ten *E.coli* isolates was resistance for tetracycline and oxytetracycline , these isolates were tested for identify the prevalence of tetracycline resistance genes tet (A). Nine out ten 9/10 (90 %) of *E. Coli* isolates were carried *tet*A gene (Figure.8). The tet (A) gene of strains were amplified by PCR with two sets of primers targeting tetracycline efflux gene (tetA).

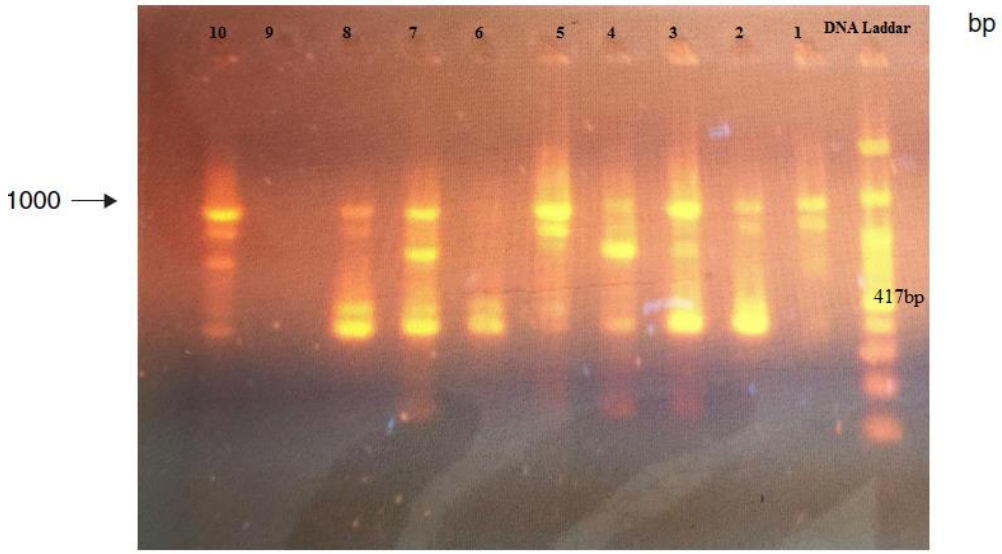


Figure.8: Strain genomic DNA profiles obtained with multiplex PCR. Shows the results for isolates of ten oxytetracycline/ tetracycline-resistant *E. coli* strains obtained from different animals. Multiple bands obtained from nine strain except isolates no. 9.

Discussion

A basic understanding of the spreading and diversity of antibiotic-resistant bacteria and their resistance mechanisms is necessary for effective prevention and control of antibiotic resistance and its dissemination. Most of the resistance determinants are found to be on mobile genetic elements, such as plasmids, transposons or integrons (Davison 1999; Rowe-Magnus et al. 2002). The prevalence of horizontal gene transfer makes certain that antibiotic resistant environmental strains deserve better investigations, especially in veterinary medicine, where the chance for humans to contact antibiotic-resistant bacterial contamination via consuming of animal product is high. In this study, totally 53 samples including 9, 37, 6 and 1 were collected from cow , sheep, goat and cat respectively. The total number of isolated bacteria were 53 isolates. The number of bacterial isolates isolated from sheep were 25, 6, 3, 1 and 1 for *E. coli, Mannheimia haemolytica, Pasteurella Multocida, Klebsiella, Proteus and Samonella* respectively. Moreover, *E. coli* was the most common isolated bacteria. The majorities of these isolates were resistance to the panel of antibiotic discs and showed multip-bacterial resistance. The resistance of bacteria to tested antibiotic revealed high percentages 81.57%, 92.1%, 84.21%, 89.47%, 94.7%, 84.21% for S, Te, C, T, P and SXT respectively. Moreover, oxytetracycline and Tetracyline showed the highest percentages 92.1%, 94.7% respectively. This result are in agreement with the results of previous studies that approved presence of multiple-drug resistance from fecal samples of sheep. (Lipsitch et al., 2002).

The results of this study also approved the presence of resistance bacteria in goat. The number of bacterial isolates were 4,1 and 1 for *E.coli Stapylococcus and Micococcus* respectively. Meanwhile, this study also approved that *E. coli* was the most common isolated bacteria. Additionally, the majorities of these isolates were resistance to the panel of antibiotic discs that used in this study. While the percentages of resistance bacteria reached to 100 % for some antibiotics. The percentages of resistance were 100%, 100%, 83.33%, 100%, 83.33% and 100% for S,Te, C, T,P and SXT respectively. These results are compatible with previous studies that reported the presence of multiple- resistance bacteria in the farm animals including goat (Chopra, Roberts, 2001). The samples and bacteria isolated from cow and from goat showed also resistance to different types of antibiotics with variations in the percentages of resistance to each type of antibiotic. The number of bacterial isolated that investigated in cow were 4, 1,1, 1 and 2 for *E.coli, klebsiella, pasteurella, Streptococcus* and *Proteus* respectively. The result of this study also approved that *E. coli* was the most common isolated bacteria as the situation in sheep and goat. The majorities of these isolates were resistance to the panel of antibiotic discs that used in this study. The percentages of resistance were 77.77%, 100%, 77.77%,100%,100% and 77.77% for S,Te, C, T, P and SXT respectively. Besides, this study showed that some isolates show 100% resistance to Te, C, T. This results are in agreement with previous studies that approved the isolation of antimicrobial resistant (AMR) bacteria from cow and its environments. This result is consistent with previous studies (Tamtam *et al.*, (2011).

Almost the vast majority *E. coli* isolated from animals were resistant to tetracycline and oxytetracycline. This might be explained by the fact that both are heavily used in the veterinary clinic for treatment result of inadvertent use. This suggests that the extent of resistance to an

antibiotic is associated with the extent of its use. The high antibiotic resistance rate of organisms isolated from animals is not a phenomenon unique to Iraqi animals. The report found that 100 % of farm *E. coli* strains were resistant to tetracycline and oxytetracycline. The present study findings, approved the increasing resistance of *E.coli* to antimicrobial agents in different countries worldwide Spain (Rose & Pedersen, 2005). One report revealed multidrug resistance(MDR) in E. coli recovered from Irish cattle. Daini and Adesemowo (2008) found the resistance of *E. coli* clinical strains from Nigeria in 54 and 88% strains against gentamicin and tetracycline respectively, which is in agreement with the current finding. The high percentage of resistance to pefloxacin (88%) and amikacin (71%), which are rarely used in the farm animals, is raising lot of questions as to why there is a high level of resistance to such antibiotics in natural non-clinical animals and how the bacteria acquired resistance against the antibacterials. If these antibiotics are to be used, it is used only for treating bacterial infections not amenable to other commonly applied antibiotics such as enrofloxacin, ciprofloxacin and gentamicin. MDR had been reported previously where in all isolates exhibited resistance to more than six antibiotics that did not differ from the findings of the present work. In the present study, only one (10%) *E. coli* isolates did not showe the presence of tetA gene that agreed with the higher percentages of tetracycline- resistant isolates. It can be assumed that this tetA negative isolates reported in this study might be encoded by other genes such as tetB, tetC and tetD or ribosomal protection encoded by tetM, tetO, tetQ and tetS genes than the gene monitored in this study. Not only did those animal hosts that presumably had continuous exposure to tetracycline have a higher percentage of tetracycline-resistant *E. coli* isolates, but also those isolates carried a greater diversity of resistance genes. Moreover, these isolates often had more than one tetracycline resistance determinant. This suggests that the human activities provides suitable environments that select for resistant strains and encourages the transfer of genetic information from unrelated bacterial species (Rahman et al., 2002). Koo and Woo (2011) have reported that 98.3% of meat-borne *E. coli* containing at least one of the tetA to tetD genes was able to transfer tetracycline resistance to a tetracycline-susceptible recipient strain of *E. coli*. Interestingly, two isolates carried both tetA and tetB, but only tetA was transferred to the recipient strain. It can be presumed that the tetA gene can be spread more easily in the environment than tetB. Antimicrobial resistance can spread to humans and animals via direct or indirect contact, consumed food/feed and through the environment. Therefore, it is important to analyze the epidemiology and mechanisms of emergence and spread of antimicrobial resistance.

In conclusion, this study approved the presence of antimicrobial resistant (AMR) bacteria that isolated from different clinical cases refereed to Al Muthanna veterinary hospital. Moreover, this study investigated the spread of resistant *E. coli* in Iraqi animals, with special emphasis on tetracycline and oxytetracycline resistant *E. coli*. This study also approved the presence of tetA resistance gene that found in 90% of the tested resistance *E.coli*. The Authors recommend to do another future study that include high number of bacterial isolates and determine another antimicrobial genes responsible for transfer the resistance between other kind of bacteria. Besides, the authors recommend to use the antimicrobial wisely and prohibited providing this products without receipt.

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