



RESEARCH ARTICLE

Molecular Identification of Some Multidrug Resistance Gram Negatives Bacteria from Clinical Mastitis Cow and Study the Antimicrobial Activity of *Thymus Vulgaris* in Basrah Provence

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ARTICLE INFO	ABSTRACT
Received: Apr 24, 2024	Bovine mastitis, udder parenchyma inflammation, causes glandular tissue pathology and milk abnormalities. These developments affect the global dairy industry economically. Due to the growing importance of Gram-negative bacteria like <i>Escherichia coli</i> and <i>P. aeruginosa</i> , clinical mastitis (CM) incidences are projected to rise. form 20 isolates that suspected as <i>Pseudomonas aeruginosa</i> and <i>Escherichia coli</i> showed that eight isolates were diagnosed as <i>Pseudomonas aeruginosa</i> and five isolates were diagnosed as <i>Escherichia coli</i> . The antimicrobial susceptibility test against eight strains of <i>Pseudomonas aeruginosa</i> showed that (85%) isolates were resistant to Tetracycline and Gentamicin and (75%) isolates for each of Chloramphenicol and Novobiocin while the antimicrobial susceptibility test against five strains of <i>Escherichia coli</i> Showed that (100%) isolates were resistance for Chloramphenicol, (80%) and (60%) isolates were resistance to Ciprofloxacin, Tetracycline and Gentamicin respectively. The current study sought to determine the antimicrobial activity of <i>Thymus vulgaris</i> against resistant <i>Pseudomonas aeruginosa</i> and <i>Escherichia coli</i> . The hot alcoholic extract and the hot water extract were found to be more effective against <i>Escherichia coli</i> than <i>Pseudomonas aeruginosa</i> , and the heated alcoholic extract was more effective than the cool alcoholic extract and the hot water extract.
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INTRODUCTION

There are numerous factors that can cause mastitis in cows, including traumatic, physiological, and allergic events. However, infectious events are the most prevalent, as the disease has been associated with over 140 distinct species of microorganisms (1). The disease leads to substantial economic losses due to expenditures related to diagnostic procedures, veterinary care, reduced productivity, and indirect costs such as early culling of animals and lower reproduction rates. (2). It is expected that the importance of mastitis increases in accordance with Gram-negative, bacterial pathogens like *E. coli* and *P. aeruginosa* (3). Thus, many cases of all clinical mastitis will be resulted (4). *E. coli* represents the most popular Gram-negative species leading to CM in dairy cattle (5). Lipopolysaccharide (LPS), an endotoxin of *E. coli*, remains active in powdered newborn formula milk due to its thermal stability at 100°C, posing a concern to formula-fed neonates (6).

E. coli infection is persistent and likely to reoccur, according to studies. In recent years, *E. coli*-caused cow mastitis has increased (7). As an opportunistic pathogen found in the environment, *E. coli* may cause zoonotic illnesses in humans and animals. Animal husbandry and veterinary clinics employ

antibiotics to treat *E. coli*-infected mastitis more effectively. *E. coli* may acquire broad-spectrum antibiotic resistance and fast variation, according to research (8).

P. aeruginosa is extensively found in the skin and intestines of both people and animals as well as in the environment (9). The rise of multi-drug-resistant (MDR) *P. aeruginosa* has been a growing problem in clinical therapy due to the rising resistance of *P. aeruginosa* strains to several medications (10). Against multidrug resistant *P. aeruginosa*, conventional antibiotics are almost useless. Cow mastitis brought on by MDR *P. aeruginosa* has drawn more attention lately and resulted in substantial financial losses for producers (11).

METHODOLOGY

Sample collection and Identification of bacteria:

The current research contained a total of 60 milk samples, which were obtained from clinical mastitic cows. Milk samples were centrifuged at 3000 rpm. Various types of agar, including blood, EMB, and MacConkey, were used to cultivate a small portion of the sediment. The next step was a 24-hour aerobic incubation period at 37 °C. The growing suspected colonies were identified morphologically and biochemically (12). Pure colonies were cultivated on brain heart infusion agar to study the structure of the growing colonies and the germs' interaction before being removed to Gram stain in order to conduct genetic identification of the bacterial strains.

Molecular detection.

Extracting genomic DNA

Isolated DNA was recovered using a genomic DNA purification kit. Electrophoresis on 0.8% Agarose revealed DNA bands under UV light (13).

Identifying Polymerase Chain Reaction (PCR)

To identify PCR, 16SrDNA primers F-5'-AGAGTTTGATCCTGGCTCAG-3' and R-5'-GGTTACCTTGTTACGACTT-3' are employed (14). The final result was electrophoresed on a DNA ladder made of 2% agarose (1000). PCR product the band size 1250 bp. Genomics were sequenced using an Applied Biosystems 3730XL at the National Instrumentation Center for Environmental Management (nicem).

Antibiotics sensitivity test

Mueller-Hinton agar plates were inoculated with *Pseudomonas aeruginosa* and *Escherichia coli* isolates and incubated overnight in nutrient broth until turbidity reached 0.5 McFarland standards. Mueller-Hinton agar was tested for antibacterial susceptibility using disk diffusion. This was accomplished by meticulously swabbing the entire surface of Mueller-Hinton agar plates after immersing a sterile swab stick in overnight nutrient broth. The test bacteria were treated with the following antibiotics: Tetracycline (10µg), Ciprofloxacin (10µg), Chloramphenicol (10µg), ceftriaxone (10µg), Gentamicin (10µg), and Novobiocin (10µg). After that, the contaminated plates were gently placed on top of the antibiotic multi disc (Oxoid), and a little amount of pressure was applied. After that, the plates were kept in an incubator at 37°C for around 18–24 hours. The zone of inhibition was measured in millimeters, and the isolates were classified as sensitive or resistant using conventional charts (15).

Preparing *Thymus vulgaris* extracts.

Hot alcohol extract .

Mix 50 grammes of *T. vulgaris* powder with 300 millilitres of 70% ethanol to make a heated alcohol extract. A rotary evaporator was used to evaporate the solution at 60°C after it had been refluxed for three days and filtered through Whatman No. 3 (16).

Cold alcohol extract .

A total of 50 grams of *T. vulgaris* leaves were combined with 3,000 milliliters of ethyl alcohol that had a concentration of 70%. After three days of agitation at room temperature, the mixture was filtered through Whattmann number 3 after being subjected to normal conditions. After being rotary evaporated at 80°C, the filtrate was moved to a Petri dish and left to dry at room temperature (17).

Hot water extract

For hot water extraction, 50 g of crushed *T. vulgaris* leaves were mixed with 300 ml of distilled water. After three days of reflux, the solution was filtered and evaporated at 60°C using a rotary evaporator (16).

Antimicrobial activity

The disk diffusion method (18) is employed to assess the antimicrobial activity. which involves the use of absorbent sterilized paper discs (9mm diameter) that have been moistened with extracts. After that, the discs are put on top of the agar. The germs grew all over the agar except where the product would stop their growth. The bacteria were spread out all over the agar. Incubation surrounding the discs revealed a definite circular zone, the inhibition zone, which evaluated the extracts' effects on *Pseudomonas aeruginosa* and *Escherichia coli*. After adjusting for the 9 mm disc diameter, Mauser vernier calipers measured and reported the growth inhibition halo diameter in mm. Everyone was tested three times.

Determining the minimum inhibitory concentration (MIC):-

The disc diffusion method, as outlined in (19), was employed to determine the minimum inhibitory concentration (MIC) of *Thymus vulgaris* extracts with a minor modification. Filter paper discs with a diameter of 9mm, which were briefly sterilized, were impregnated with varying concentrations of *Thymus vulgaris* extracts (50mg/ml, 100mg/ml, 200mg/ml, and 300mg/ml) and stored at 40°C for 24 hours. After inoculating 0.1 ml of bacterial culture into Muller-H agar medium and allowing it to reach a turbidity standard of 0.5 McFarland overnight, prepared discs were placed on the agar surface. The lowest concentration (MIC) that prevents observable bacterial growth was established.

RESULTS

Identifying *Pseudomonas aeruginosa*

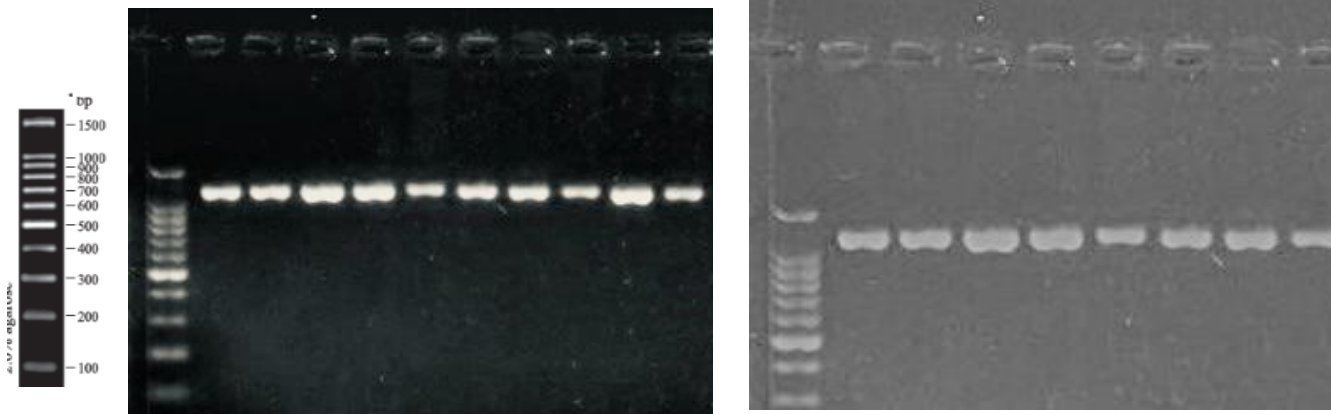
Using blood agar and MacConkey agar medium, twenty isolates were suspected of being *Pseudomonas aeruginosa* from sixty milk samples in total. Although the isolates did not digest lactose sugar, they produced β -hemolysis on blood agar and grew on MacConkey agar. To stain Gram, thin smears from one colony were prepared on glass slides. In Gram's staining, the isolated *P. aeruginosa* was Gram-negative and rod-shaped.

Identifying *Escherichia coli*

From a total of sixty milk samples, twenty tow isolates were suspected as *Escherichia coli* on EMB agar and MacConkey agar media. The isolates produced metallic sheen appearance and show pink color on MacConkey agar with ferment lactose sugar. For Gram's staining, thin smears from a single colony were made on glass slides. The morphology of isolated *Escherichia coli* revealed Gram-negative, rod-shaped bacteria in Gram's staining.

Polymerase Chain Reaction (PCR) for bacterial identification

Ten isolates of *Escherichia coli* and eight isolates of *Pseudomonas aeruginosa* were selected from the total suspected isolates. The required band of 16S rDNA for the isolate, as well as an electrophoresed ladder in the 1250bp region, was obtained by using a universal primer, as illustrated in figure (1).



The findings of sequencing for 20 isolates that suspected as *Pseudomonas aeruginosa* and *Escherichia coli* showed that eight isolates were diagnosed as *Pseudomonas aeruginosa* and five isolates were diagnosed as *Escherichia coli* as shown in table (1)

Bacterial species	Identical to strain	Accession
<i>Pseudomonas aeruginosa</i>	strain KI6.12	MT113103.1
<i>Pseudomonas aeruginosa</i>	strain HL2	KF413420.1
<i>Pseudomonas aeruginosa</i>	strain Iraq.PA-5	KX963360.1
<i>Pseudomonas aeruginosa</i>	strain ks10	OM009294.1
<i>Pseudomonas aeruginosa</i>	strain 64	KT302405.1
<i>Pseudomonas aeruginosa</i>	strain Huaian_3_1	MN314598.1
<i>Pseudomonas aeruginosa</i>	strain KI5.3	MT113101.1
<i>Pseudomonas aeruginosa</i>	strain PR2	KT949921.1
<i>Escherichia coli</i>	AAE	LC764402.1
<i>Escherichia coli</i>	strain E21	KY780356.1
<i>Escherichia coli</i>	strain TEM 112	MT912572.1
<i>Escherichia coli</i>	E008406	CP104647.1
<i>Escherichia coli</i>	strain EGE	KY655105.1

Antibiotics sensitivity test:

The results of the antimicrobial susceptibility test for five antibiotics, Tetracycline (10µg), Ciprofloxacin (10µg), Chloramphenicol (10µg), ceftriaxone (10µg), Gentamicin (10µg) and Novobiocin, against eight strains of *Pseudomonas aeruginosa* showed that 7 (85%) isolates were

resistant to Tetracycline and Gentamicin and 6 (75%) isolates for each of Chloramphenicol and Novobiocin, while there were 6 (75%) and 5 (62.5) isolates are sensitive for each of Ciprofloxacin and ceftriaxone, respectively fig (2).

The results of the antimicrobial susceptibility test against five *Escherichia coli* strains

Showed that 5 (100%) isolates were resistance for Chloramphenicol, 4 (80%) and 3 (60%) isolates were resistance to Ciprofloxacin, Tetracycline and Gentamicin respectively. while there were 4 (80%) isolates were intermediate susceptibility for Novobiocin and 4 (80%) isolates were sensitive for ceftriaxone, fig (3).

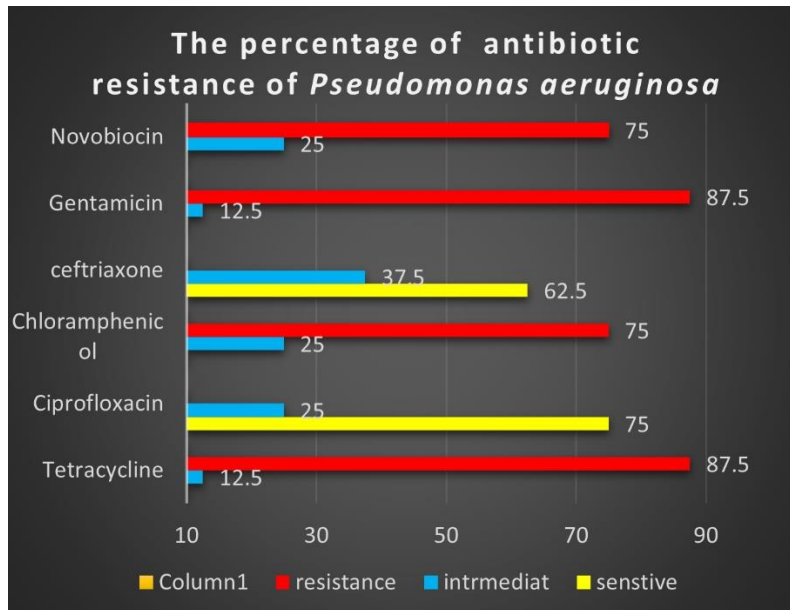


Fig (2) The percentage of antibiotic resistance of *Pseudomonas aeruginosa*

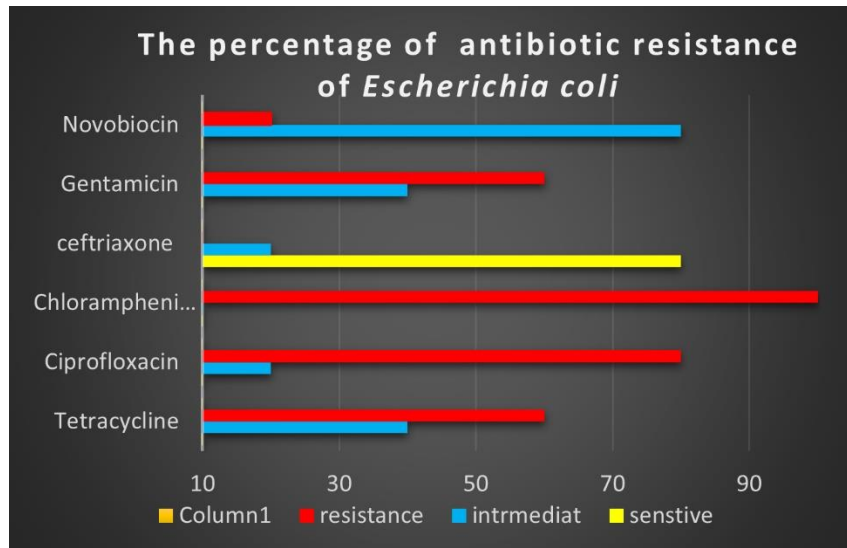


Fig (3) The percentage of antibiotic resistance of *Escherichia coli*

Antimicrobial activity of *Thymus . vulgaris* extracts against *Pseudomonas aeruginosa* and *Escherichia coli* :-

The results of the antimicrobial activity test for *Thymus vulgaris* extracts showed that the hot alcoholic extract is more effective than the cold alcoholic extract and the hot water extract, which did not show any effectiveness. The extracts are more effective on *Escherichia coli* than *Pseudomonas aeruginosa* as shown in table 2

Table (2) Mean of Diameter (mm) of the Inhibition zones Induced by the *Thymus . vulgaris* extracts \pm standard deviation

Microorganisms	hot alcoholic extract	cold alcoholic extract	hot water extract
<i>Pseudomonas aeruginosa</i>	24.5 \pm 1	15 \pm 0.154	-
<i>Escherichia coli</i>	25.5 \pm 4.932	17.5 \pm 2.203	-

Determining minimum inhibitory concentration (MIC) against *Pseudomonas aeruginosa*.

The results of the minimum inhibitory concentration study against *Pseudomonas aeruginosa* showed that the minimum inhibitory concentration was 100 mg/ml for the hot alcoholic extract with an inhibition diameter of 13 mm, while the minimum inhibitory concentration for the cold alcoholic extract was 300 mg/ml with an inhibition diameter of 12 mm fig 4.

Diameter of inhibition (mm) of Minimum Inhibitory Concentrations against *Pseudomonas aeruginosa*

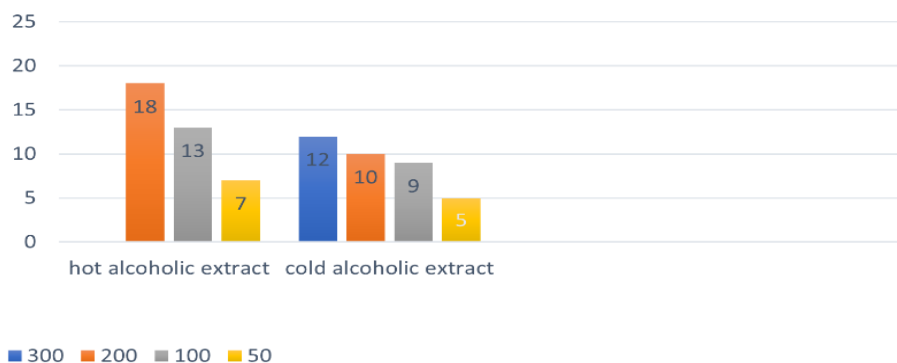


Fig (4) diameter of inhibition (mm) of minimum inhibitory concentration against *Pseudomonas aeruginosa*

Determination of minimum inhibitory concentration (MIC) against *Escherichia coli*.

The results of the minimum inhibitory concentration study against *Escherichia coli* showed that the minimum inhibitory concentration was 100 mg/ml for the hot alcoholic extract with an inhibition diameter of 18 mm, while the minimum inhibitory concentration for the cold alcoholic extract was 200 mg/ml with an inhibition diameter of 14 mm fig 5.

Diameter of inhibition (mm) of Minimum Inhibitory Concentrations against *Escherichia coli*

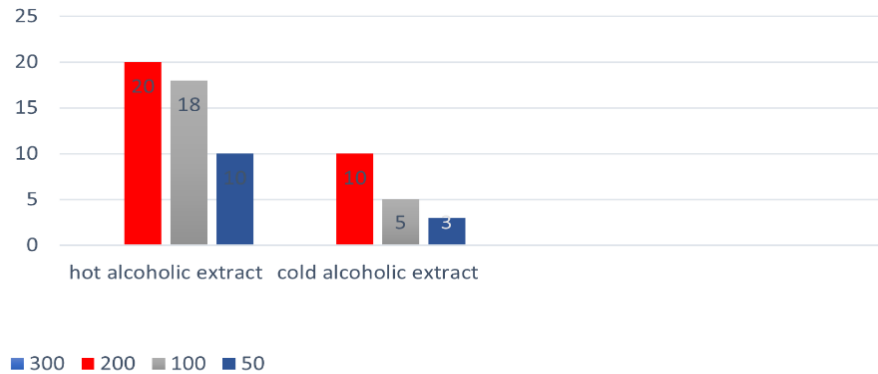


Fig (5) diameter of inhibition (mm) of minimum inhibitory concentration against *Escherichia coli*

DISCUSSION

Gram-negative bacterial pathogens including *Escherichia coli* and *P. aeruginosa* are expected to become more important in mastitis. This group of bacteria accounts for a disproportionately large number of cases of clinical mastitis (CM) (4). The most common Gram-negative bacterium that causes CM in dairy cattle is *Escherichia coli* (5). The percentage of *P. aeruginosa* recovered from milk was more than (5.4%), compared to milk samples obtained from cows in Pungal that had subclinical mastitis (20). The results of the antimicrobial susceptibility test conducted against eight strains of *Pseudomonas aeruginosa* revealed that (85%) of the isolates were resistant to gentamicin and telacycline, and (75%) of the isolates were resistant to both chloramphenicol and novobiocin. Additionally, the test (21) revealed that 50% of *Pseudomonas aeruginosa* are resistant to gentamycin and CP.

Research has consistently shown that antibiotic treatment of coliform mastitis in calves, whether it is clinical or subclinical, has little to no effect (22). This is proved through increasing antimicrobial-resistant isolates and the occurrences of recurrent and persistent mastitis cases that are resulted from the same *E. coli* isolate. Thus, the *E. coli* isolated from the mastitis-affected cattle's milk resist various kinds of antibiotics (23).

This investigation aims to identify the *Thymus vulgaris*'s antimicrobial activity against resisting *P. aeruginosa* and *Escherichia coli*. Concerning the antibacterial activity tests' findings, it is revealed that the hot alcoholic extract considerably impact the cold alcoholic and hot water extracts. The extracts are more active against *Escherichia coli* than *Pseudomonas aeruginosa*. The lowest inhibitory concentration for the hot alcoholic extract was 100 mg/ml in the *Pseudomonas aeruginosa* minimum inhibitory concentration research using a 13-mm inhibition diameter. The minimum amount of cold alcoholic extract that halted the reaction was 300 mg/ml, and the obstruction was 12 mm. The *Escherichia coli* minimum inhibitory concentration investigation found that the hot alcoholic extract had a minimum inhibitory concentration of 100 mg/ml and an inhibition diameter of 18 mm, whereas the cold extract had 200 mg/ml and 14 mm. the findings of this work agree with those of (24).

CONCLUSION

According to the findings of the current investigation, it is concluded that hot alcoholic extract has more impact than the cold alcoholic extract. Moreover, the hot water extract significantly effect the *E. coli* rather than *P. aeruginosa*. The researcher recommends that in addition to the evaluation of extract in vivo as an alternative antibacterial activity, the bioactive compounds should be isolated and purified from *T. vulgaris* extract.

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