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Determination genes of Beta-lactamase in *E. coli* and histological state for appendicitis patients in Basra province

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Abstract

The current study has been done to determine the pathogenic bacteria that are associated with appendicitis. This study includes ninety samples of removed appendices taken from patients who were diagnosed with appendicitis infection by specialized doctors in general Basrah Hospital and Al-Sadir Teaching Hospital for the period between September 2023 and June 2023. The percentage of samples that gave positive culture was 80 (88.9%), while 10 (11.1%) of these samples had negative culture. The study found 15 different bacterial isolates, with *Escherichia coli* being the most common at 80 (44.9%), while other species appeared in lower percentages. *Shigella dysenteriae 14 (7.9%), Salmonella enterica typhi 10 (6.5%), and other strains with lower percentages* Laboratory diagnosis for blood samples included an estimate of total WBCs and found that 31% of patients have natural WBC values, while the other patients have high values.

The most common bacteria showed that all isolates were resistant to most antibiotics used in the test, especially for the lactam group, and the isolates of *E. coli* were multi-resistant to antibiotics.

The plasmid profiles of *E. coli* isolates were investigated to study the correlation between plasmid profiles and antibiotic-resistant markers, and results from agarose gel electrophoresis revealed that all *E. coli* isolates contain one plasmid band.

This study includes the detection of some genes that encode beta-lactamase enzymes in *E. coli*, which are responsible for multiantibiotic resistance. These genes were loaded on plasmid DNA for ten isolates and found that 5 (50%) of isolates have *bla*TEM gene, 40% have *bla*CTX gene, and 1 (10) has *bla*SHV gene.

This study also considered the general appearance of appendix samples: some of them are enlarged and surrounded by vesicles, some with fibrous walls and ulcerated with mixed colors, and then examined the histological changes. The study showed changes in the histological structure of the excess, including extensive congestion of blood vessels and veins in the serosa

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and subserosal layers and increased amounts of diffuse lymphoid tissue in the layers of the appendix walls.

Keywords: Appendicitis, Beta-lactamase, Plasmid DNA, histological examination.

1. Introduction

The appendix is a blind-ending tube with mucosal, sub mucosal, muscular, and serosal layers. It arises from the cecum in the right iliac fossa. They have an average length of 7.5–10 cm [1]. Appendicitis is an inflammation of the appendix. Once it starts, there is no common effective therapy, and it requires emergency surgery to prevent complications [2, 3]. Acute appendicitis can sometimes progress to gangrene [4].

Many bacterial species have been diagnosed in appendicitis patients in many countries, such as *Escherichia coli* and beta-hemolytic Streptococci [5, 6]. *Pseudomonas aeruginosa* [7], *Staphylococcus aureus*, *Enterobacter* spp. [8], and *Bacteroides fragilis* [9].

Appendicitis affects about 260,000 operations in the United States every year [10]. While there were no reports in Iraq, because most studies focused on appendicitis diagnosis, the aims of this study were to determine:

- Isolation and identification of the most common bacteria associated with appendicitis.
- The determination gene of beta-lactamases in *coli* and the histological state of appendicitis patients in Basra city.

2. Methods

2.1 Collection of Samples

During the period from September 2023 to June 2023, 90 specimens of surgically removed appendicitis were collected, and 3 ml of blood was removed from the patients in the operation rooms of Basra General Hospital and Al-Sadr Teaching Hospital. Then the blood was placed in a tube with anti-coagulated material. A 5 mm-thick sample was taken and placed in the formalin solution at a concentration of 10% for histological study. In addition, the remaining pieces of appendicitis were placed in sterile glass tubes containing the Brain Heart Infusion (BHI) broth. The patient's information (name, age, sex, and place of residence) was registered. Both the appendectomy and blood samples were soon transferred to the laboratory for bacteriological testing and the rest of the tests [11].

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2.2 Bacteriological culture for the appendix

After taking notes on the length, diameter, and general appearance of the appendectomy models, 70% of the alcohol solution was submerged for several minutes for the purpose of sterilization. Then, it was longitudinally transplanted with a sterile surgical incision and a swab from that piece [12] and cultured on the following media: MacConkey Agar Medium, Blood Agar Base Medium, Chocolate Agar Medium, Salmonella-Shigella Agar (S-S Agar), and Mannitol Salt. The dishes were incubated at 37 °C for 24–84 hours, and the appendix was kept after a swab in 10% formalin was taken for tissue examination [5].

Storage of bacteria: the bacterial isolate was kept in the refrigerator for one month after being stored at 37 °C for 24 hours on a BHI slant [8].

2.3 Diagnosis of Bacteria

2.3.1 Macroscopic and Microscopic examination

Bacterial colonies were initially identified in terms of shape, texture, and odor, and the identification of negative and gram-positive isolates was based on observation of growth and absence of growth on the MacConkey medium, recording of the isolating fermented or non-fermented colonies, observation of *E. coli* EMB medium, and differentiation of *Salmonella* bacteria from *Shigella* bacteria on SS Agar medium, as well as the use of the gram stain technique to differentiate the positive bacteria from the negative.

2.3.2 Biochemical Tests

All the isolates under study were subjected to following biochemical tests [13, 14]

1- Oxidase test.
2- Catalase test.
3- Urease test.
4- Citrate utilization test.
5- Indole test.
6- Triple sugar iron agar.
7- MR-VP test.
8- Detection test of the capsule.

2.3.3 API-20E test

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- Preparation of bacterial suspension. Prepared the bacterial suspension by taking a pure single colony by loop from at the age of 18–24 hours and then placing it in a 5 ml of saline solution with a concentration of 0.85 NaCl

- To compare turbidity with McFarland solution, mix well to homogenize and use immediately after preparation. 0.5.

- Using the pipette, the bacterial suspension is distributed in the tubes of the test strip.

- After adding the oil, they covered the tape and incubated at a temperature of 37 °C for 18–24 hours. After incubation, the reagents were added, and the results were identified based on the Analytical Profile Index, which is included with the diagnostic system.

2.3.4 Total white blood cell (WBCs) count

The Neubauer chamber count and the Turk's solution, consisting of glacial acetic acid (1.5 ml) or methyl violet (1 ml), were used to calculate the total number of white blood cells. The method is summarized as follows:

- Used a pipette of the white blood cells to draw the blood to the 0.5 mark after leaving the first droplets, and then diluted it using Turk's solution to the mark 11.

- Mixed diluted blood and then poured on the slide and left for two minutes to stabilize the W.B.C.

- Then the number of white blood cells is counted as in the following equation: The number of white blood cells per cm3 equals the number of cells calculated in four large squares multiplied by 50.

2.3.5 Isolation and Extraction of DNA Plasmid DNA Using Kit

- Transferred 600 microliters from the growing broth to 5 mL abendrof tubes.

- Add 100 microliters of cell lysis buffer and mix. It should be changed from opaque to blue, indicating complete decomposition.

- 350 microliters of coolant solution (4–8 °C) was added and mixed by turning the tube. The sample color turns yellow, and a yellow deposit is formed.

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- The sample operates in the centrifuge at its maximum speed for three minutes.
- Transferred the filtrate (900 microliters) to the small tubes containing the filter and installed it in the collection tubes, and the machine worked at maximum speed for 15 seconds.
- Leave the filter in the collection tube, and place the small tube in the same collection tube.
- Added 200 microliters of Endotoxin Removal Wash to the small tube and put it in the centrifuge for 15 seconds at maximum speed.
- Added 400 microliters of the washing solution to the small tube and put it in the centrifuge at maximum speed for 30 seconds.
- Transferred the small tube to a clean 1.5-ml ebendrof tube, then added 30 microliters of the elution buffer directly to the small tube and left it for 1 minute at room temperature.
- Centrifuge at high speed for 15 seconds to remove the DNA plasmid, then cover the ebendrof tube and store the DNA plasmid at -20 °C.

2.3.6 Electrophoresis for DNA plasmid

All plasmid DNA samples isolated from bacterial isolates are electrophoresed as follows:

- 0.25 g of solid agarose dissolved in 25 mL of TBE buffer was heated on a hot plate until boiling, and then allowed to cool to 45–50 °C.
- Added 0.5 microliters of the aqueous bromide to the agarose and slightly cooled it to homogenize.
- Poured the cold agarose into the transfer plate after closing the sides of the board with adhesive tape and placing the drill to make wells in the special place.
- Left the gel for 20–30 minutes to solidify completely.
- Filled the transfer basin with the TBE until the gel was 3 mm higher.

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- Put one microliter of loaded stain in one well of the DNA molecular loader (1 kb DNA loader).

- Placed the transfer basin cover and passed electric current with a voltage difference of 60 volts for one hour.

2.4 PCR Programs

2.4.1 PCR Programs for determine the *bla*$_{TEM}$

The table following describe this determine [16]

Steps	Temperature	Time	No. of Cycles
Initial Denaturation	94 ˚C	12 min	1
Denaturation	94 ˚C	60 sec	30
Annealing	55 ˚C	60 sec	
Extension	72 ˚C	90 sec	
Final Extension	72 ˚C	10 min	1

2.4.2 PCR Programs for determine the *bla*$_{CTX-M}$ gene

The table following describe this determine [17]

Steps	Temperature	Time	No. of Cycles
Initial Denaturation	94 ˚C	7 min	1
Denaturation	94 ˚C	50 sec	35
Annealing	50 ˚C	40 sec	
Extension	72 ˚C	1 min	
Final Extension	72 ˚C	5 min	1

2.4.3 PCR Programs for determine the *bla*$_{SHV}$ gene

The table following describe this determine [17]

Steps	Temperature	Time	No. of Cycles
Initial Denaturation	96 ˚C	5 min	1

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Denaturation	96 ˚C	1 min	35
Annealing	60 ˚C	1 min	
Extension	72 ˚C	1 min	
Final Extension	72 ˚C	10 min	1

2.4.4 The primers used to identify genes

The primers used in this study as following [18]

Product size (bp)	**Primer sequence (5′→3′)**	**Primer name**	**Target**
1355-1155	ATAAAATTCTTGAAGACGAAA	TEM-F	*bla*TEM
	GACAGTTACCAATGCTTAATC	TEM-R	
800	TG GT TAT GC GTTATATTCG CC	SHV-F	*bla*SHV
	GGTTAGCGTTGCCAGTGCT	SHV-R	
900	TCTTCCAGAATAAGGAATCCC	CTX-F	*bla*CTX-M
	CCGTTTCCGCTATTACAAAC	CTX-R	

2.5 Preparation appendages for histological study

The histological study is achieved by using method of landfill with paraffin wax [19].

2.6 Statistical Analysis

Statistical Package for Social (SPSS 11) is used as statistics program.

3. Results and discussion

3.1 Bacteriological study

The bacteriological study included 90 appendicitis from patients in the Basra General Hospital and Al-Sadr Teaching Hospital for the purpose of isolating the aerobic and anaerobic facultative bacteria. The percentage of samples that gave a positive result for bacterial transplantation was 80 (88.9%), while samples showing no bacteria were 10 (11.1%). Fifteen bacterial species were isolated based on cultural characteristics, microscopic examination, and biochemical tests, as well as KIT for the diagnosis of the Enterobacteriaceae.

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The results in Figure 1 showed that *E. coli* is the most common gram-negative bacteria. This is expected to have the highest isolation rate compared to other species. These results are consistent with other studies [20].

The variation in the types of isolated bacteria in the present study may be due to many causes: the environmental variability of the bacterial species, the location of the swab, and the immunity of the infected person [21], which showed that any bacterium found in the human digestive tract can cause appendicitis [22], or may be appendicitis due to anaerobic bacteria, and there may be reasons other than bacterial [20].

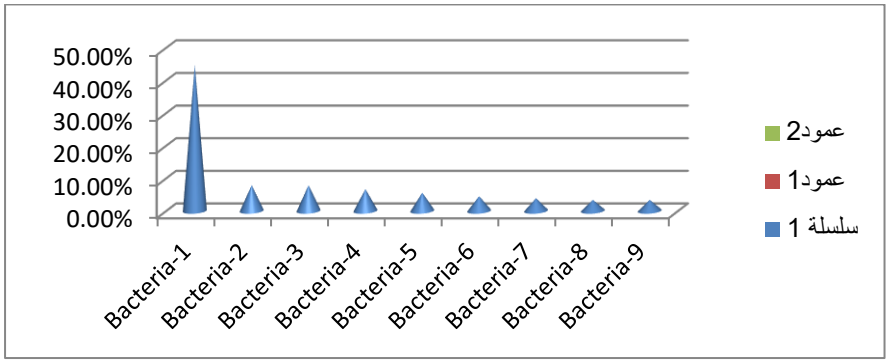

Figure (1): Percentages of isolated bacteria from appendectomy samples.

1-*E. coli;* 2-*Shigella dysenteriae*; 3-*Staphylococcus* spp.; *4-Streptococcus* spp.; 5-*Salmonella enteric typhi; 6-Pseudomonas aeruginosa; 7-Morganella morganii; 8-Klebseiella pneumonia; 9-Neisseria* spp.

3.2 Determination of plasmid content in *E. coli*

The results of the electrolysis on the agarose gel showed that all isolates of *E. coli* (10 isolates) contain one plasmid band and have a 1 KB DNA ladder. (Figure 2) illustrates the plasmid pattern of plasmid DNA samples isolated from the antibiotic-*resistant E. coli* bacteria, where the number of antibodies resistant to them has reached 8–13 antibiotics out of 20 types of antibiotics.

In the current study, bacterial isolates were selected, all of which showed multiple resistance to antibiotics, for the isolation and determination of plasmid patterns in order to determine the relationship between the presence of plasmids in bacterial isolates and multiple resistance. The current study revealed the detection of the encoded genes of some large-spectrum beta-lactase enzymes in *E. coli* isolated from appendicitis patients using polymerase chain reaction technology (PCR) using *bla*CTX, *bla*SHV, and *bla*TEM. The results of the molecular diagnosis

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showed that all the tested isolates had at least one of the above-mentioned genes. The study showed a high presence of 50% TEM, 40% CTX, and 10% SHV.

The results of the electrolysis of polymerase chain reaction (PCR-4) products showed (Figure 3) that five out of ten isolates had TEM-wide-spectrum enzymes that might be resistant to *E. coli* antibodies to lactam antibodies to these enzymes. The first enzymes found in the intestinal family carry their genes on plasmids [23].

TEM enzymes are given beta-lactamase resistance to penicillin, ampicillin, and first-generation cephalosporin antibiotics. These enzymes are responsible for 90% of the ampicillin resistance in bacterial isolates *like E. coli.* The results of the current study agreed with those of Study [24]. The current study showed that two isolates out of four isolates had 50% TEM beta-lactase enzymes. This result was also agreed with by a local study in Najaf, which showed that 14 isolates (66.6%) of 21 isolates of *E. coli* were able to express this gene [25]. However, this finding differed from another local study in Hilla, which recorded a single isolate (20%) out of 5 isolates that had the TEM beta-lactase gene. Also, the current study found that four isolates (40%) out of the 10 isolates producing high-spectrum beta-lactamase enzymes possess a CTX-M gene through the polymerase chain reaction products (Fig. 4). This study agrees with the study [26]. In addition, they found a single isolate (10%) out of 10 isolates producing high-spectrum beta-lactamase enzymes possessed a *bla*SHV gene (Fig. 5) by using specific primers. The results were consistent with the local study in Najaf, which showed that 6 isolates, or 30% of the 20 isolates, revealed the

The emergence of a SHV gene [27]. This contrasted with the study [24], which found that 3 (75%) out of 4 isolates of *E. coli* produce wide-spectrum bectalactime enzymes.

The production of these enzymes is carried out by the *bla*SHV gene. K. pneumonia isolates contain mainly SHV enzymes, while these enzymes are also present in *E. coli* bacteria [26]. It was recently reported that 15.1% of E. coli isolates from clinical samples in Turkey were able to produce SHV enzymes using the polymer chain reaction technique [28].

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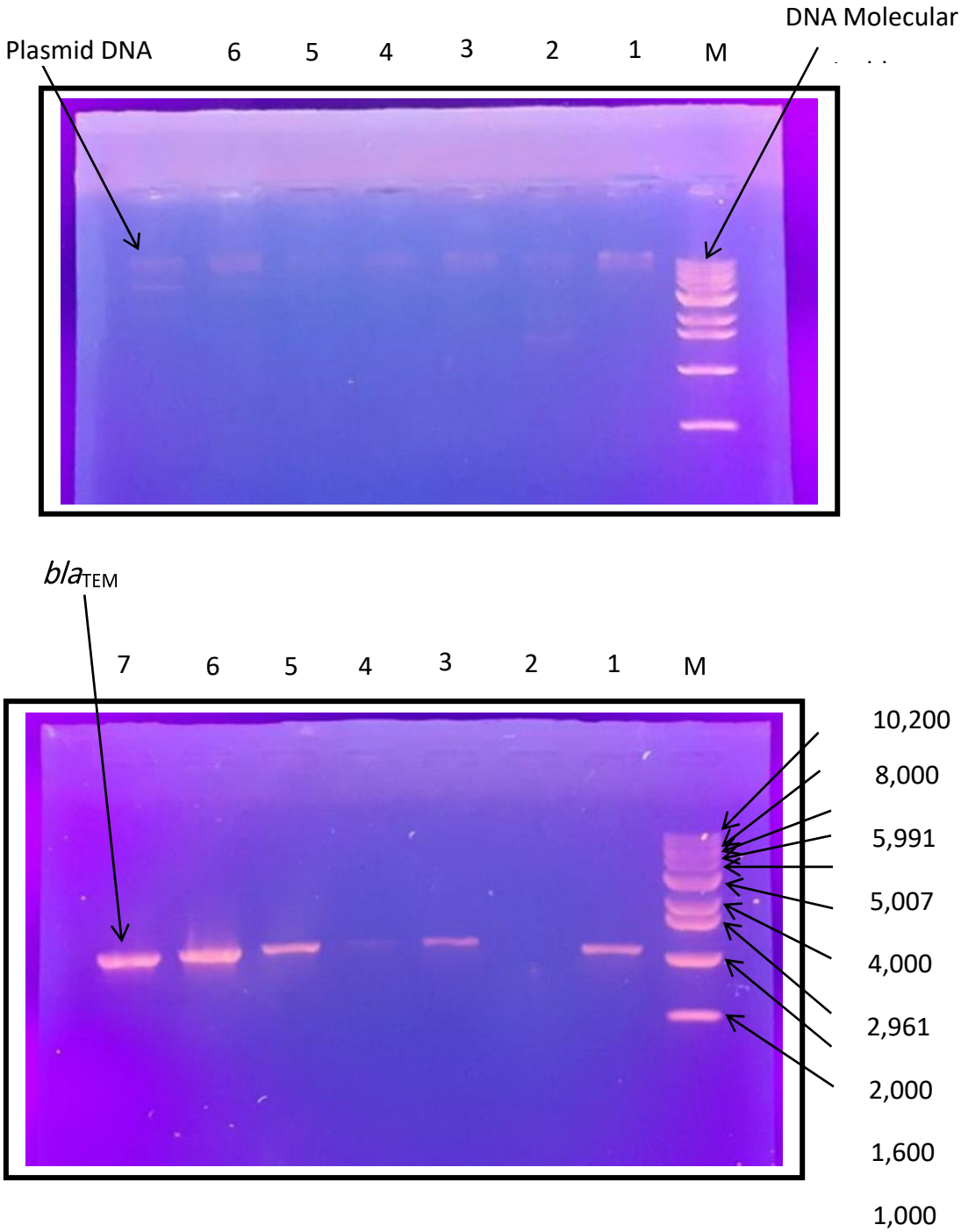

Figure (2): Gel electrophoresis using agarose gel (1%).

(1-7): Plasmid pattern of multiple antibiotic resistant of *E.coli* isolates.

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Figure (4): Gel electrophoresis using agarose gel (1%).

(1, 3, 5, 6, 7): the positive result of the *bla*TEM gene

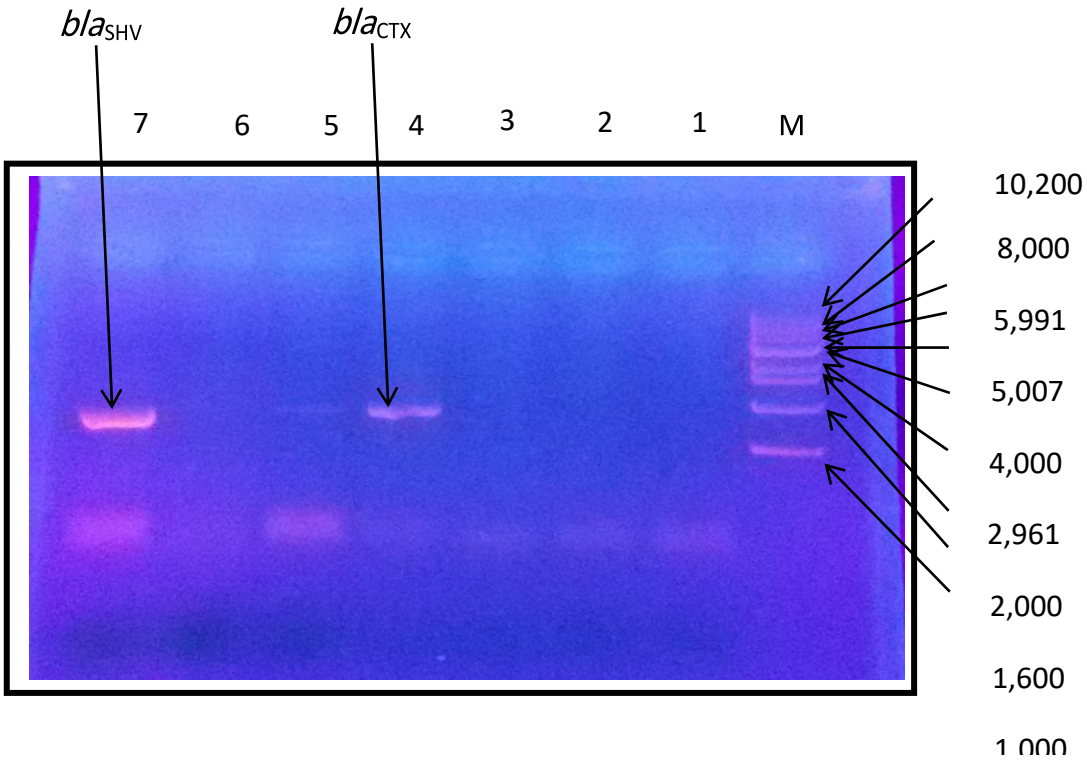

Figure (5): Gel electrophoresis using agarose gel (1%).

(4): Positive result for *bla* CTX gene. (7): Positive result for *bla* SHV gene

(1, 5, 6): Positive result for *bla* CTX gene.

3.3 Total W.B.C. Count:

Table (1): The total number of white blood cells.

M ± SD	Range	Age group
10968.75 ± 4955.638	21000 – 4500	20 – 10
10606.25 ± 3677.403	19000 – 4600	30 – 20
11814.29 ± 7745.198	25500 – 4100	40 – 30

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8100 ± 2687.006	18000 – 6200	50 – 40
8000 ± 2828.427	10000 – 6000	60 – 50
N.S.	N.S.	

M: Average, SD: Standard Deviation, N.S: No significant differences

The results of the white blood cell count (Table 1) showed clear changes in the number of white blood cells in the appendicitis patients. There were no significant differences in the values of white blood cells, while minor changes were observed among the age groups (10–20 years old). The results showed an increase in the total number of white blood cells in the first and second age groups, but not for all the patients. In addition, there was no significant increase in the number of white blood cells in a number of cases (31%).

The results are contrary to other studies that showed about 80–85% of adult patients with appendicitis had an increase in the number of white blood cells of more than 10,000 cells per milliliter, and less than 4% of patients with appendicitis had less than 10,000 cells per milliliter. WB.C. numbers increase during inflammation, but not all cases may have a normal white blood cell count in patients with appendicitis bursting. There are some studies that have demonstrated W.B.C. elevation during inflammation (Table 1). This can be explained by the occurrence of physiological events at these ages affecting the level of W.B.C. and the rate of survival compared with normal adults [15].

The WBC counts are particularly sensitive in children and the elderly with acute appendicitis. But it may be unreliable in spite of the high level of W.B.C. because it may be useless if there is a decline in value. In addition, the body's natural immune defenses play a role in this increase because of the incomplete specialized immune system, especially in children, and this is consistent with the paper [29].

3.4 Morphology of the Appendix

There is a clear difference between an inflamed appendix and a natural appendix in their general appearance. The inflamed appendix appeared swollen, as is observed in the change in the size of the appendix, with pale and clear blood vessels on their surfaces. In addition, the color of flaming inflorescences strongly turns to a red or blue color and may be a grayish green color, especially in the follicles, which are exacerbated by the condition of the gingaria (Figure 6, a). Light pink (Fig. 6, b). On the surfaces of some infected vertebrates, fatty vesicles were found along the length of the excess, distorted in shape and the typical diameter, which made

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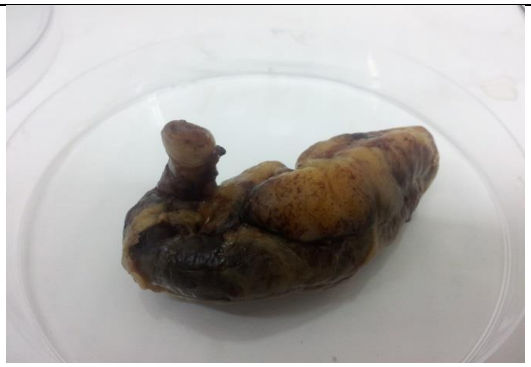
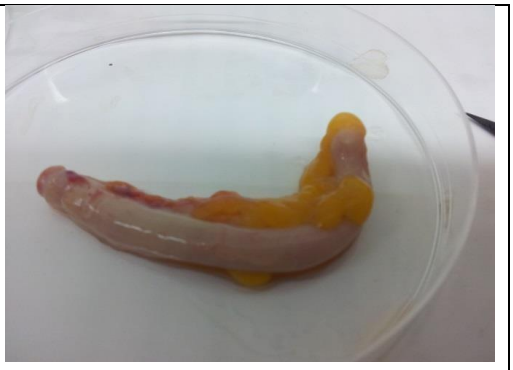
it difficult to distinguish the direction of the excess (Fig. 6, c). It is noted that in some polyps, their shape and size are normal but characterized by the presence of fatty vesicles around them (Fig. 6, d).

It was noted that there was a difference in the attempt to cut the wall appendix longitudinally, as the walls of the inflamed appendix were rough and ulcerated with the transformation of part of the fabric or all of it to the fibrosis layer (Figure 6, e). The spread and number of ulcers and fibrosis on the outer surface of the appendicitis is a clear indication of the severity of the inflammation. This variation in the phenotypic changes of appendicitis may be due to the type and severity of inflammation [30].

Macroscopic examination of this study revealed the presence of a fossilized stone inside the appendix. It also revealed that the repeated case in four samples were pebbles of different sizes, causing the excess cavity appendix to be filled, which provides favorable conditions for bacteria to multiply and attack excess tissue appendicitis, causing the inner lining to be filled with pus and lacerated (Figure 6, f). The presence of a stone inside appendicitis raises controversy in predicting its reason. They are probably formed as a result of the calcification of food waste with digestive secretions and purulent material as a result of the secretions of the mucous layer in the cavity of appendicitis because of the obstruction, partially or completely excess, which then increases the pressure on the excess wall and causes acute inflammation of the kind. People are also affected by the food's nature because it encourages low-fiber foods to be more feces-like. It contains a large proportion of them and thus increases the likelihood of clogging the excess cavity [11].



A): inflamed appendicitis converts it color to red to blue.



(B): Appendicitis looks normal in white or pink color and lack of clarity Vessels.

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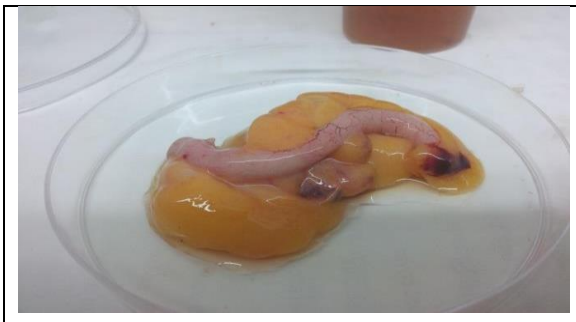
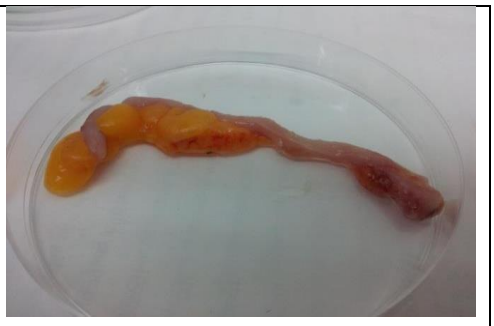
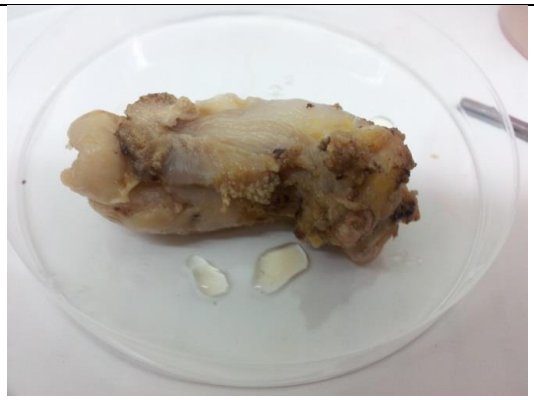


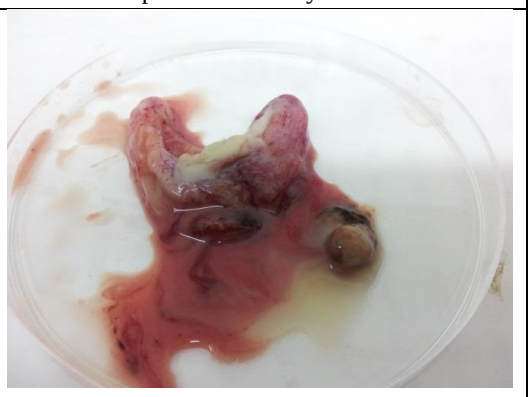
(C): An inflamed appendicitis is surrounded by fatty vesicles.



(D): Appendicitis looks natural appearance, presence of fatty vesicles around it



(E): Appendicitis with rough, ulcerated walls.



(F): Existence of a fossilized inside the appendicitis.

Figure (6): The phenotypic examination of the appendix.

3.5 Histological study

The histological examination of the appendicitis is aimed at two purposes: first, to enhance and confirm the diagnosis of the inflammatory condition, especially if no specific changes are observed in some of the samples and second, to detect additional causes that may be obtained during laboratory tests [31].

The microscopic examination of tissue sections showed changes in the tissue structure, starting from the epithelial layer and extending to the tissue layers one by one. It was observed in some appendages that the excess cavity was significantly enlarged. Vascular changes were also observed, such as venous vasoconstriction and expansion of the cavities in the mucous and subcutaneous layers (Figure 7). This result agrees with the findings of [32, 33]. As a result of the activity of the bacteria, the pus accumulates in the cavity and then the sub mucosal layer (Fig. 8). This is in line with [22]. The expansion of the excess cavity causes the gases produced by the inflammatory bacterial species as metabolites to increase the pressure on the internal excess walls.

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The histopathological study showed an increase in the amount of lymphoid tissue diffused in the mucous layer and sub mucosal as well as the infiltration of lymphatic cells into the plate Lamina Peoria (fig. 9) and the spread of other inflammatory cells such as macrophages and neutrophil cells (fig. 10). Also, the study showed histological changes can be explained as a result of this case for an immune response to infection, as excess lymphatic participation in the defense against infection pathogens, including bacteria. Bacteria are the first steps in the epithelial layer of tissue injury, followed by the invasion of bacteria and other layers that possess virulence factors to give rise to resistance [20].

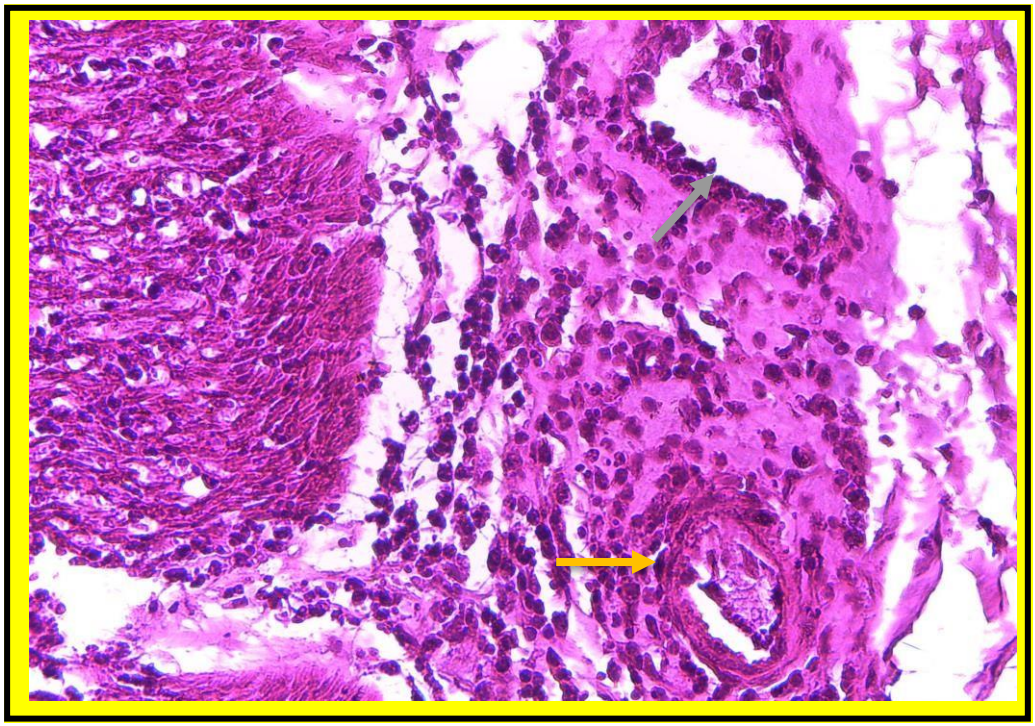

Figure (7): A longitudinal section in appendicitis tissue showing blood vessel congestion and the proliferation of inflammatory cells. (Pigmented with H and E), (343.1X).

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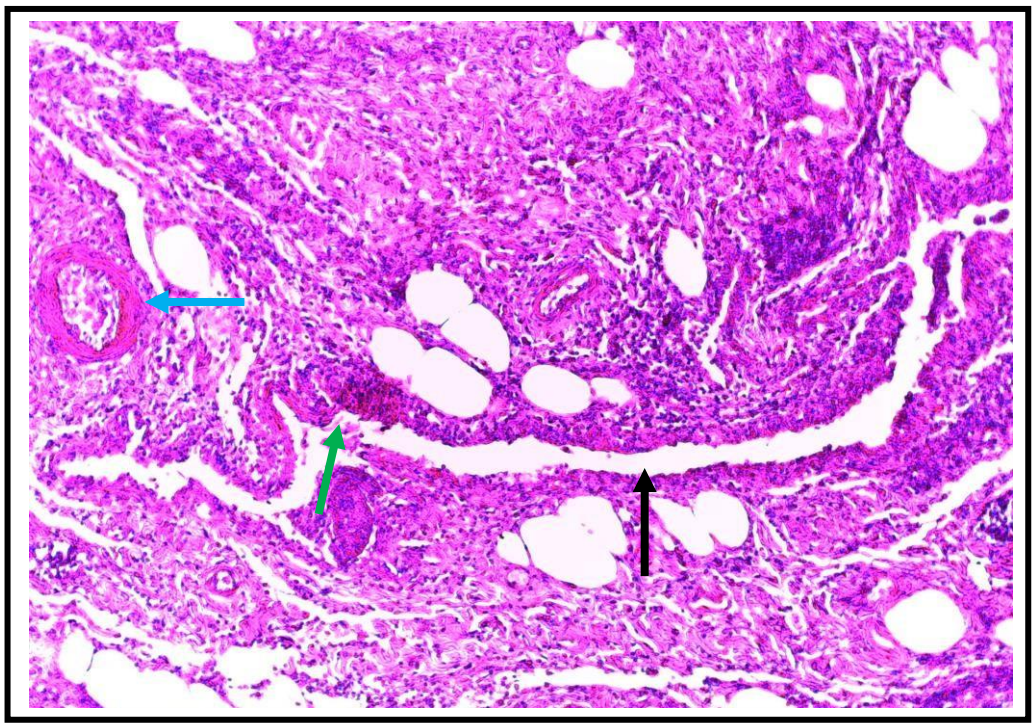

Figure(8): A longitudinal section in appendicitis tissue showing the appearance of inflammatory secretions And a number of elongated glands with ulceration .(Pigmented with H and E), (343.1X) .

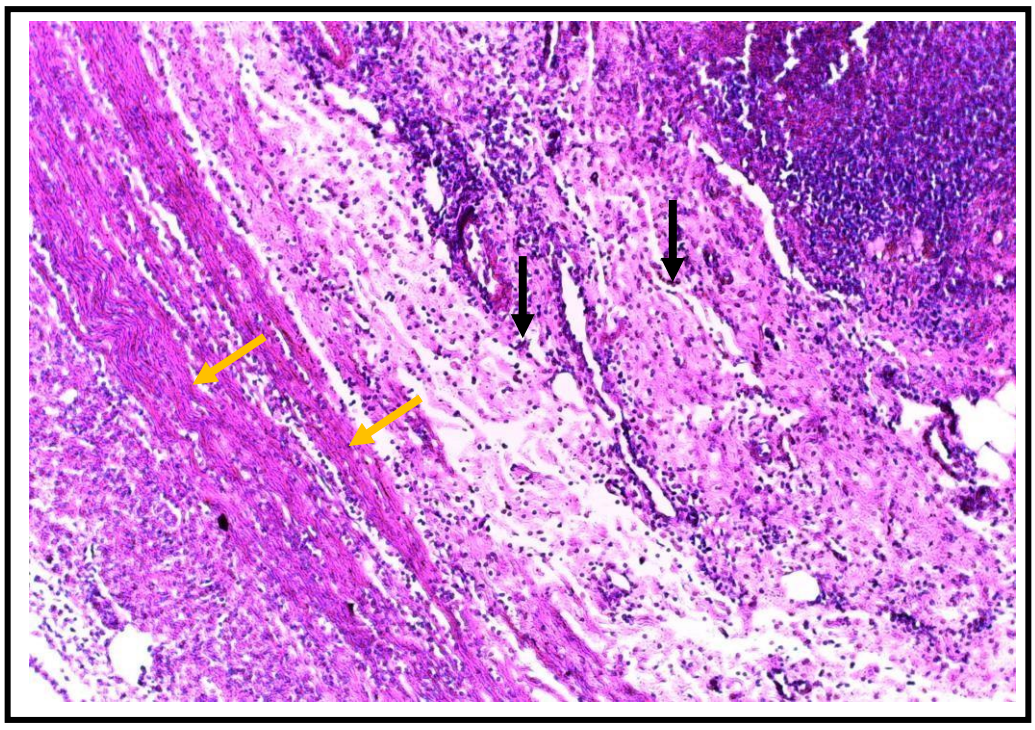

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Figure (9): A longitudinal section in appendicitis tissue showing part of the original layer filtering through inflammatory cells and bands of smooth muscles.

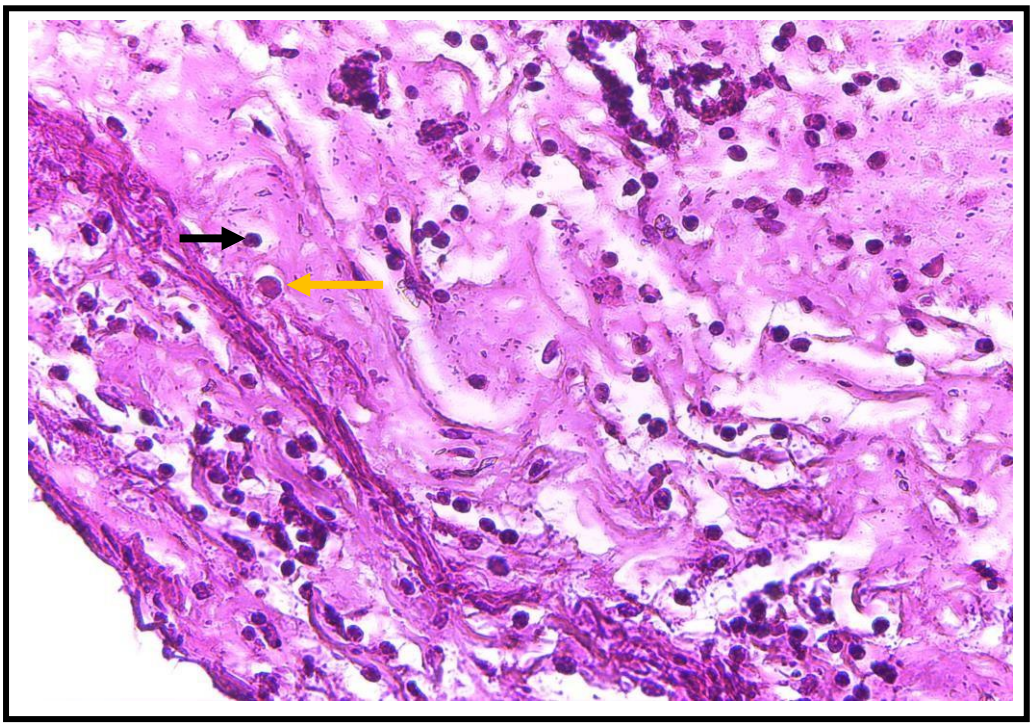

Figure (10): A longitudinal section in appendicitis tissue appear inflammatory cells, multiple Macrophage, Neutrophil, and fiber.

4. Conclusion

Bacteria are the predominant cause of appendicitis, accounting for 88.9%. It is possible to rely on the criterion of the total number of white blood cells as a primary auxiliary method for detecting infection in children and the elderly. Resistance of the most common bacteria to most antibiotics used in the treatment of appendicitis. The worm before and after the operation and in the event of its explosion. Bacteria possess enzymes that help them resist antibiotics, and these enzymes are present in genes carried on plasmid DNA. The histological study showed noticeable histological changes in the tissue of the appendix, and they were diagnosed as chronic and acute conditions.

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