

Histopathological Examination of Appendicectomy Specimens: Common Detected Pathologies and Negative Appendectomy Rate in Patients Undergoing Appendicectomy

Dr. Zahraa Majid Hussein AL-Bawi^{1*}, Dr. Maha tawfeeq jasim ², Dr. Sarah Dheyaa Saeed Alkattan³

ABSTRACT

Author's InformationActa1. MBCHB CABMS pathology.appoDiprcpath of Royal College ofthatHistopathology, National centerdiagof teaching laboratories,NegBaghdad medical city complex,gross

2. MBCHB CABMS Pathology, National center of teaching laboratories, Baghdad medical city complex, Baghdad, Iraq

3. MBCHB CABMS pathology, National Center of Teaching Laboratories , Baghdad Medical City Complex , Baghdad ,Iraq.

Corresponding author: Dr. Zahraa Majid Hussein AL-Bawi zahraamajidalhassun@gmail.com

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Acute appendicitis is one of the commonest surgical emergency worldwide, appendectomy or appendicectomy among the commonest surgical procedures that performed on daily practice. Histopathology is the gold standard for diagnosis of gold standard of all examined specimens after appendectomies. The Negative appendectomy result from excision of normal appendix. Intra-operative gross examination and pre-operative imaging and other investigations still have some diagnostic limitations compared to histopathology. The objective of our study is to assess the results of histopathological examination of appendicectomy specimens and the common detected pathologies and estimate the negative appendectomy rates in patients undergoing appendicectomy. We included 117 specimens of patients after appendectomies. Standard laboratory histopathological protocols and procedures were followed in collection and processing of these specimens. Results of our study showed that most patients were at young age (11 - 30) years with predominance of males in a male to female ratio of almost 1.4 to one. Negative appendectomy rate (NAR) was 17.1% and was comparable to the accepted global ranges. In conclusion, conducting histological analysis of the appendix specimens provides valuable clinical insights in conjunction with operational findings, a significant higher prevalence of appendicitis among individuals younger than 30 years. Females, older age patients and those with high preoperative WBC count exhibited a higher negative appendectomy rates. Hence, in order to prevent the oversight of any clinically significant and manageable conditions, it is imperative to incorporate a systematic histological investigation into the routine analysis of all appendicectomy specimens.

Keywords: Appendicitis, Appendectomy, histopathological examination, Negative appendectomy

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1. INTRODUCTION

On daily practice in surgery, the most common surgical emergency is acute appendicitis and can lead to serious complications such as peritonitis, ileus and even mortality that makes appendectomy among the commonest surgical procedures that performed (1). The incidence of appendicitis varies worldwide, however, globally it is estimated to be 233 per 100,000 population. On the other hand, the lifetime risk ranged between 6.7 to 8.6%. From other point of view, the incidence of appendicitis declined in the western countries in the midtwentieth century, but there still a higher incidence rate and increasing trend in some countries (1–3). Since hundreds of thousands of appendectomies are performed annually this translates to thousands of negative appendectomies, hence, it is important to find a costeffective method to reduce the prevalence of unnecessary appendectomies, without thereby increasing the morbidity and mortality of the appendiceal pathological process as such. When surgeons diagnose appendicitis, they must carefully consider the potential consequences of both false positive and false negative diagnoses. On one hand, there is a danger of unnecessary removal of a healthy appendix in patients with atypical signs and symptoms. On the other hand, there is a risk of appendix perforation if a conservative approach is used and the condition is not promptly treated (4–6). Ideally, both the prevalence of perforation and negative appendectomies could be decreased by increasing diagnostic accuracy. Ultrasound and computed tomography (CT) have emerged as the commonest modalities for diagnosing acute appendicitis, owing to their enhanced precision (7,8). Furthermore, these modalities have contributed to promote the antibiotic administration in the management of appendicitis. It is advised that patients who do not exhibit high-risk CT abnormalities initially undergo conservative antibiotic care, with surgical intervention being considered if antibiotic treatment is ineffective(9,10). The identification of acute appendicitis poses a significant obstacle, even for seasoned surgeons, as characteristic manifestations are observed in just 60% of cases. Moreover, other medical disorders exhibit similar symptoms to acute appendicitis, particularly in women, leading to challenges in diagnosis and a higher incidence of negative appendectomies (11,12). Therefore, histological examination continues to be regarded as the most precise approach for verifying the diagnosis of appendicitis as the gold standard of all examined specimens after appendectomies (7). In addition, histopathological examination (HPE) of appendectomy materials has the potential to identify further atypical features, including parasite infections, endometriosis, or granulomatosis (13). The use of early appendectomy as a treatment for clinically suspected appendicitis has led to a reduction in negative health outcomes. However, it is well acknowledged by both surgeons and pathologists that a notable part of the appendices that are removed do not exhibit any signs of inflammation. The incongruity between the clinical manifestation and the absence of definitive morphological alterations is perplexing. Several recent investigations have indicated that certain individuals may exhibit presenting symptoms with an underlying inflammatory basis (14). The precise origins and mechanisms behind the development of appendicitis are inadequately comprehended (14). The pathophysiology of acute appendicitis is believed to involve two main factors: blockage of the lumen, leading to distension and disruption of circulation, and invasion of the appendix wall by microorganisms. It is widely accepted that the latter occurrence is the last step in the development of acute appendicitis (14,15). In histopathology studies of specimens after appendectomy, the primary diagnostic characteristic of acute inflammation was the identification of extravascular polymorphs inside the epithelium, lamina propria, or muscle layers. The appendix wall exhibited evident visibility, accompanied by significant destruction of the mucosa. Additionally, there was a notable presence of neutrophil infiltration, which extended extensively throughout the submucosa and into the muscularis externa. The ganglia had a heightened prominence, observed not only in the interstitial spaces between the circular and longitudinal muscle layers but also in the deeper regions inside the muscle layers (14,16,17). A substantial quantity of nerve fibers and Schwann cells were discovered to be extensively dispersed throughout the submucosa and the muscularis externa, with particular prominence in the circular muscle layers. There was a notable increase in both the quantity and dimensions of ganglia seen in the muscularis externa and submucosa. The smooth muscle fiber is seen as distinct muscular bundles that are separated by inflammatory exudates composed of a significant quantity of polymorphonuclear cells, together with other types of inflammatory cells (14,16,17).

Histopathological examination of surgical specimen are common in daily practice, and pathologists reported many reports and discover different valuable insights about the diagnosis. The histological analysis of specimen of cases after surgery for acute appendicitis fulfills two objectives; Firstly, it enables the confirmation of acute appendicitis diagnosis, particularly in cases when it is not apparent during the surgical procedure. Furthermore, the utilization of histological analysis can reveal other diseases that may not be readily apparent during the initial intra-operative gross examination. These findings have the potential to significantly impact the future clinical care of the patient. Negative specimens for acute appendicitis are valuable in ruling out acute appendicitis, however, potential explanation for symptoms needs other investigations if symptoms continue. In instances of negative appendicitis, it is common for patients' symptoms to resolve following the surgical procedure. There has been a suggestion that in such instances, there might potentially sub-clinical manifestation of appendicitis (18,19). According to previous studies, the intra-operative detection rate for various forms of appendiceal tumors was shown to be less than 50% (20). A significant proportion of surgeons failed to identify aberrant pathology findings in a majority of patients, necessitating further examination, investigations and treatment, this reflects the importance of histopathological assessment of appendectomy specimens (21) where a wide range of lesions and pathologies detected by histological testing of appendectomy specimens , for instance, According to the literature, hyperplastic polyps, Mucinous cystadenomas, tumors, like carcinoid lesions . For instance, the incidence of adenocarcinoma was found to be 0.24% in some studies. Moreover, the majority of benign tumors are effectively treated with the surgical procedure of appendicectomy alone. Nevertheless, there are some instances in which the need for right hemicolectomy is evident. Hence, It is recommended that all individuals diagnosed with appendiceal tumors undergo regular follow-up, as there is a potential risk of developing a subsequent malignancy in around 20% of these cases. Additionally, Some lesions in negative appendectomies may pass unnoticed if the specimen had not been submitted for standard histological study (18). The histopathological study of surgical specimens is done for several reasons: to give a specific diagnosis, to obtain information on the prognosis, to guide therapeutic conduct, for medico-legal purposes, and as quality control and feedback regarding treatment and other decisions made by the surgeons. According to the clinical guideline of the College of American Pathologist, certain surgical specimens must be sent for pathology study (22). Nevertheless, researches that examines the advantages of analyzing appendectomy specimens are scarce particularly in our country. Consequently, several available facilities, opt to submit all surgically removed specimen after appendectomy for histological examination. Hence, the objective of our current study is to evaluate the information obtained from the histological study of Specimens obtained after appendectomies for adult Iraqi patients in comparison to clinical and operative findings and also to estimate the negative appendectomy rates.

2. METHODOLOGY

Study design and setting:

The present study is a cross-sectional study conducted during the period from January 1st 2022 to June 31st 2023, spanning a duration of almost 16 months. The evaluation encompassed a histopathology examination of the specimens referred by surgeons which were collected from patients undergoing appendicectomy during the study period. Pathology reports pertaining to patients who had undergone appendectomy. The key variables considered in this analysis were the age and sex of the patients, as well as their histological diagnosis and any relevant operative findings. Demographic data, preoperative imaging findings, surgical approach, and histopathology reports were collected for each patient. The clinical diagnosis of appendicitis was often established by surgeons possessing a minimum of 2 years of expertise in the field of general surgery. Specialist supervision was limited to instances in which challenges arose during diagnosis or intraoperative procedures. Histological evaluation was conducted by a team of pathologists (Researchers).

Study Population:

The present study included the clinical data of a total of 123 patients who had appendicectomy for suspected acute appendicitis and requests for histopathology examination. Histological data were subsequently obtained. 6 patients were excluded due to incomplete pre or operative data and the net sample was 117 cases.

Inclusion criteria:

- 1. The study involved Iraqi individuals who underwent appendicectomy due to a clinical presentation that was indicative of acute appendicitis.
- 2. Patients aged older than 10 years and of both genders
- 3. Specimens of the appendix that were removed during the appendicectomy procedure and were sent for histopathology study.

Exclusion criteria:

- 1. Appendicular masses that were effectively treated through conservative methods,
- 2. Incidental appendicectomy during operations for other pathologies
- 3. Cases with history of proved diagnosed malignancy in abdominal organs
- 4. Patients who received chemo or radiotherapy
- 5. Patients with chronic inflammatory diseases

6. The study did not include gangrenous and perforated appendices due to significant structural damage to the lamina propria and submucosal tissue.

Specimens collection and processing

Specimens were received from the operation theater with a histopathology study requests. All specimens were sent for conventional histopathological evaluation. Samples were promptly fixed in 10% buffered formalin for preservation before they are sent to the pathology laboratory.

A thorough evaluation was assured for all pathology requests accompanying the specimens and operating notes of atypical cases. The objective was to identify any indications that the surgeons had detected the presence of suspected pathologies in the appendix based on their intra-operative gross examination. The clinical relevance of the aberrant results was assessed by reviewing the patient notes to identify the clinical significance of our findings.

Standard procedures for processing the specimens was followed in accordance with a procedure established by our hospital.

The sectioning of the specimens were carefully performed and the sections at the tip, body, and base were obtained. Afterwards the sections subjected to examination by the pathologists (researchers). The final report contains information about both macroscopic and microscopic observations. Paraffin embedding of tissues was followed. Pieces with a thickness of 3 μ m were cut and stained with eosin and hematoxylin stain. When necessary, special stains such as periodic acid Schiff and Ziehl Neelsen were used (23).

A finding was deemed to have clinical significance if it necessitated more follow-up investigations, such as staging CT, colonoscopy, biopsy, or further surgical intervention, or if it had an impact on the patient's prognosis.

Definitions.

• Acute appendicitis: is characterized by inflammation that affects all layers of the appendix or pus present inside its lumen.

• Periappendicitis: is described as inflammation that affecting only the layers of the appendix wall and serosa is the most affected layer, while the mucosa remains unaffected.

• Incidental appendicectomy: the removal of appendix during other operations or surgical procedures in patients when acute appendicitis is not the primary indication of surgery

• Negative appendicectomy: When the appendix removed while the subsequent histological analysis of the excised tissue reveals absence of an inflammation, tumors, or infectious agents, or alternatively, the presence of periappendicitis.

Study Outcome:

The main primary outcome of our study is describing the histopathological findings of all appendectomy specimens. Our secondary outcomes include the distribution of histopathological findings and detected pathologies in relation to age and sex of patients as well as estimation of negative appendectomy rate and its distribution across age and sex of patients and the correlation with other variables

The histopathological diagnosis were subsequently categorized as either positive or negative for acute inflammation. The positive cases, characterized by neutrophil infiltration, were further categorized into three distinct categories.

Study analysis:

All requests were carefully reviewed and all data were reported in a computerized database using an excel sheet. Then transferred to Statistical Package for social sciences software for statistical analysis. Scale variables were tested for normal statistical distribution and presented as mean ± standard deviation (SD). Nominal variables expressed as frequencies and percentages. intra-operative gross examination and preoperative ultrasonography findings were compared to histopathology. Validity parameters for both intra-operative gross examination results and ultrasonography were calculated according to the following equations (24):

Sensitivity = TP/ (TP + FN)

Specificity = TN / (TN +FP)

Accuracy = (TP + TN) / (TP + TN + FP + FN)

Where: TP : True positive, TN: True negative, FP: False positive and FN: False negative

3. RESULTS

A total of 117 specimens belong to 117 patients who were operated on for suspected acute appendicitis were enrolled in this study. The mean age of the studied group is 27.3 ± 8.9 (range:11-62) years. Males were relatively dominant (58.1%) compared to females (41.9%) with a male to female ratio of almost 1.4 to one (**Table 1**). All patients presented with abdominal pain, however, migratory pain reported by 38 patients (32.5%). nausea and vomiting in 69.2%, anorexia in 26.5%, fever in 16.2% and abdominal distension and mass in 1.7% for each, (Table 2). The mean preoperative white blood cell (WBC) count was $12.9 \pm$ 3.1 (x 109 /L) and only 21.4% of the cases had WBC count of 10 (x 109 /L) or less. Preoperative ultrasonography examination revealed findings that in favor acute appendicitis in 74 patients (63.2%) while the remaining 43 (36.8%) cases did not show any findings that consistent with acute appendicitis. Hence the negative appendicitis rate in ultrasound was 36.8%, (Table 3). Schematic distribution of the studied group according to the final histopathology studies is shown in (Figure 1). Histopathology confirmed acute appendicitis in 89 cases (76.1%) and while the histopathology picture showed other lesions or normal appendix in 28 cases (23.9%). According to histopathology, completely normal appendix reported in 20 specimens giving a negative appendectomy rate of 17.1%, (Figure 2). Other pathologies incidentally documented by histopathology were 8 (6.8%), these 8 cases were 3 appendiceal diverticulitis, 2 granulomatous appendicitis, and 3 patients had carcinoid, mucinous and parasitic lesions for each. Furthermore, we inserted some histopathological slides of our patients to show Histopathological appearance of normal appendix (Figure 3), acute appendicitis (Figure 4) and a case of carcinoid tumor in appendix (Figure 5). Further analysis was performed for the comparison of clinical diagnosis against histopathology study which revealed that clinical diagnosis confirmed acute appendicitis and agreed histopathology in 86 cases (True positive) while rule out 23 cases as normal appendix of them 20 appeared normal on histopathology (True negative), according to these findings, clinical diagnosis and decision had a sensitivity of 96.6%, specificity of 71.4% and accuracy of 90.6% to confirm true acute appendicitis, (Table 4). Additionally, we also compared ultrasound vs. histopathology, according to this comparison, ultrasound produce a sensitivity, specificity and accuracy of 61.7%, 53.6% and 59.8%, respectively (Table 5). Finally, we assessed the relationship between negative appendectomy rates according to histopathology with patient's age, gender, WBC count and comorbidities. Using bivariate correlation analysis, Pearson's and Spearman's tests) we found a significant direct association between age and NAR, i.e. NAR was higher in older patients and increased with advancing age. NAR was significantly higher in females (R = 0.518, P<0.001). Preoperative WBC count significantly higher in patients with negative appendectomies, where a direct (positive) correlation was found (R = 0.722, P. value < 0.001). Presence of comorbidities had very weak association with NAR and the correlation was statistically insignificant (R= 0.096, P. value = 0.318), these finidings are summarized in (**Table 6**).

Variable		No.	%
Age	11 - 20	42	35.9
	21 – 30	35	29.9
	31 – 40	19	16.2
	41 – 50	13	11.1
	> 50	8	6.8
	Mean (SD)	27.3 (8.9)	-
Gender	Male	68	58.1
	Female	49	41.9
	Ratio: 1.39 : 1.00	-	-

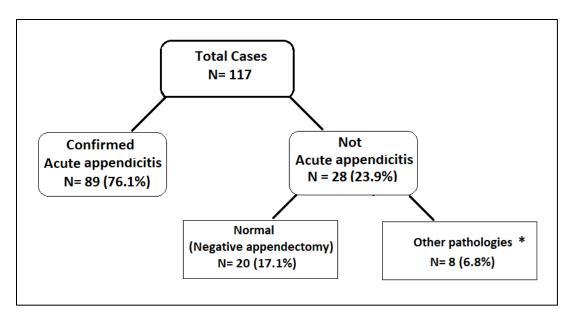
Table 1. Age and gender distribution of the studied group (N=117)

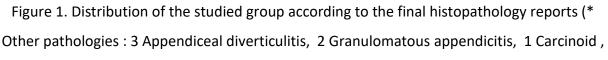
Symptoms	No.	%
Abdominal pain	117	100.0
Migratory pain	38	32.5
Nausea and vomiting	81	69.2
Anorexia	31	26.5
Fever	19	16.2
Abdominal distension*	2	1.7
Abdominal mass	2	1.7

 Table 2. Presenting symptoms of the studied group (N=117)
 117

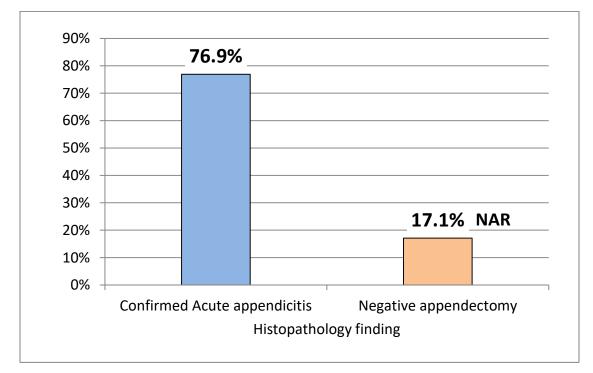
Table 3. Preoperative White blood cell (WBC) count and ultrasonographyfinding of the studied group

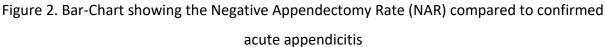
Variable		No.	%
WBC count (x 10 ⁹ /L)	≤ 10	25	21.4
	11 - 15	41	35.0
	16 - 20	45	38.5
	> 20	6	5.1
	Mean (SD)	12.9 (3.1)	-
Ultrasound finding	Positive	74	63.2
	Negative	43	36.8





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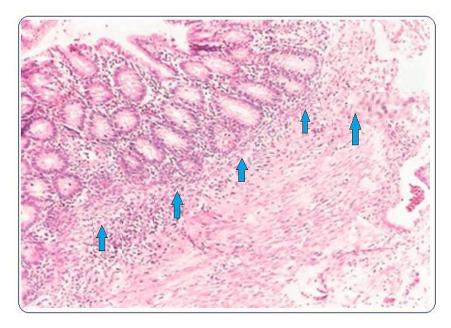


Figure 3. Histopathological appearance of normal appendix (arrows refer to normal mucosa

and sub mucosa)

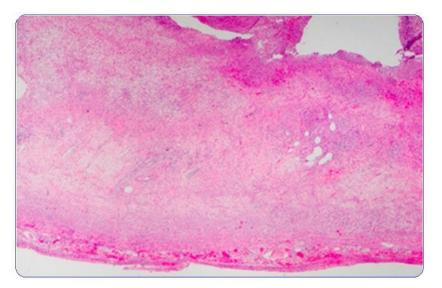


Figure 4. Histopathological appearance of appendix in case with acute appendicitis

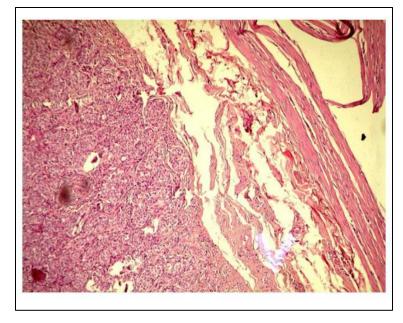


Figure 5. A photo of a Slide showing Carcinoid tumor in appendix (Hematoxylin and Eosin x

40)

Clinical diagnosis	Histopathology		Total
	Acute appendicitis	Negative	Total
Acute appendicitis	86	8	94
Negative	3	20	23
Total	89	28	117

 Table 4. Comparison of clinical diagnosis against histopathology study

Sensitivity: 96.6%. Specificity: 71.4%. Accuracy: 90.6%.

Ultrasound finding	Histopathology		Total
	Acute appendicitis	Negative	Total
Acute appendicitis	55	13	68
Negative	34	15	49
Total	89	28	117

Table 5. Comparison of Ultrasound against histopathology study

Sensitivity: 61.7%. Specificity: 53.6%. Accuracy: 59.8%.

Variable	R	P. value
Age (older age)	0.294	0.022 sig
Gender (Female)	0.518	<0.001 sig
WBC count elevated > 10 ⁹ /L	0.722	<0.001 sig
Comorbidities (present)*	0.096	0.318 ns

Table 6. Results of bivariate Pearson's and Spearman's tests for the correlation betweenNAR and other variables among the studied group

*Hypertension, Diabetes mellitus, or others. sig: significant, ns: not significant

4. DISCUSSION

In the discipline of Pathology, similar to other medical specialties, the financial implications and effort associated with certain practices prompt an examination of established routines. Consequently, there is a growing consensus to advocate for a patient-centric approach in laboratory work, tailoring processes to the unique characteristics of individual patients (25). It has been suggested that a significant proportion, ranging from 25% to 40%, of laboratory tests may lack necessity. There are several factors that contribute to the increased demand for Pathology. These factors include the need for improved diagnostic accuracy, pressures from healthcare administrators and physicians, patient attitudes and feedback, as well as concerns over potential legal actions. (26,27). Some researchers have proposed that a macroscopic examination may be sufficient for resection specimens of cecal appendages, as long as it does not reveal any changes other than those caused by the inflammatory process that led to the resection. In such cases, only specimens that appear suspicious or uncertain would be sent to the pathology laboratory (25,27). Historically, the histological studies of resected specimens from different surgical procedures spanning for more than a century (28), nonetheless, there still a debate on the justification of histological examination to be routinely performed for all specimens obtained from appendectomies, as the presence of incidental abnormalities may be clinically insignificant or have no impact on the subsequent patient care (18,27). The appendix exhibits considerable diversity in its microscopic characteristics. Based on a range of research, the prevalence of diagnoses other than normalcy and inflammation in routine microscopic examinations is estimated to be between 0.1% and 4.2%. (29,30). Different studies have advocated for the discontinuation of systematic histopathological examination of some surgical specimens due to questionable therapeutic relevance or a high cost-benefit ratio. Furthermore, findings may not provide further valuable insights and are deemed superfluous (18). Additionally, the College of American Pathologists considered that, in the absence of gross abnormalities, it is not necessary to study surgical specimen (31). From other point of view, some centers often sent specimens for histological examination only if they exhibit macroscopic pathologies on surgical procedures while other centers with good facilities opt to submit all surgically removed specimens for histological examination. Nevertheless, there is a scarcity of research that examines the advantages of examining tissues obtained from appendectomies. The aforementioned practice possesses the capacity to overlook significant diagnoses that could later impact patient care (27,29,32). In spite of the notable progress made in surgical techniques throughout the last century, the identification of acute appendicitis remains a challenging task for medical practitioners, particularly when dealing with young female (33). Histopathology is considered the gold-standard method for diagnosing acute appendicitis. The determination of diagnoses for all surgically removed specimens is achieved by conducting histological examinations on appendectomy specimens (34). The pathology report holds considerable significance not only in cases of acute appendicitis but also in circumstances where incidentally diagnosed diseases are unexpectedly discovered (35). However, there is a lack of established criteria regarding the routine submission of all appendices for histopathology. Nevertheless, a considerable number of appendiceal tumors are typically identified through examination of appendectomy specimens. Histological confirmation of the diagnosis is necessary for surgeons and medical centers to find negative appendectomy rates and to identify the factors that may contribute to lower accuracy in clinical diagnosis or unnecessary surgical procedures, in our country few histopathological studies that concern with analysis of specimens of after appendicectomy (33).

Our objectives is to assess the value of histopathological studies in identification of pathologies observed in all specimens obtained from appendectomy procedures. Additionally, assessment of incidence of the appendicitis according to age and gender. Moreover, we aimed to assess the negative appendectomy rate and the validity of ultrasonography in detection of acute appendicitis. Hence we examined 117 surgical

specimen were examined and the final histopathological reports were documented. The gross pathologies recognized by the surgeon and ultrasonography reports were compared against histopathology. Our findings showed that majority of patients were of young age (11-30) years, and there incidence of acute appendicitis was higher in males than females in a ratio of almost 1.4 to one, these finding were not unexpected, where the previous studies reported close findings to ours (23,34,36). An earlier Iraqi studies found that younger age and female gender increase the likelihood of getting acute appendicitis (33,37,38). Abdominal pain was the presenting symptom in all patients, the second commonest symptom was Nausea and vomiting followed by Migratory pain Anorexia and others, also WBC count was elevated in majority of cases, these are consistent with the clinical picture of acute appendicitis (1,3,17,34). Ultrasound findings were positive and consistent with acute appendicitis in 63.2% only. Final histopathological reports revealed a total of 89 confirmed acute appendicitis and 20 normal appendix while 8 cases had incidentally discovered as nonacute appendicitis with other pathologies. Compared to clinical diagnosis and gross evaluation of the surgeon, the negative appendectomy rate was 17.1%, this rate falls within the accepted range of 10 - 20% (39). Our reported NAR was lower than that reported by Jolayemi et al. (36) who found a NAR of 22.4%. Multiple studies have demonstrated a diverse range of rates, spanning from 6.1% to 34.2%, with a higher prevalence observed among females. Hence, it is imperative to investigate for other causes of abdominal pain in females, particularly when the appendix appears to be in a normal state following surgical intervention (39). In the current study we compared the clinical operative diagnosis and ultrasound findings from one side against the histopathology findings as gold standard and found that operative finding was better than ultrasound and had good sensitivity, specificity and accuracy than ultrasound, nonetheless, operative finding was not highly specific and false positive rate still high. However, the NAR was within the accepted range. From other point of view, histopathology successfully identified other 8 pathologies that were missed by gross diagnosis and ultrasound. Hence we suggest to send all appendectomy specimen for histopathology studies. Furthermore, we assessed the correlation between NAR and other variables, we found that older age, female gender and higher preoperative WBC count were significantly associated with higher NAR, while comorbidities were not. Almost similar findings reported in previous studies in local and international setting (4). In order to prevent the oversight of any clinically significant and manageable conditions, it is imperative to incorporate a standardized practice of histological analysis for all appendicectomy specimens. With the era of CT scan, the higher NARs are no longer accepted however, CTscan is not widely available in all of our centers, and ultrasound studies are neither highly sensitive nor highly specific and lower accurate (40–45),

5. CONCLUSIONS

The current study documented that conducting histological analysis of the appendix specimens provides valuable clinical insights in conjunction with operational findings, thereby warranting its inclusion in all instances of acute appendicitis. We demonstrated a significant prevalence of appendicitis among individuals in the adolescent and young adult age groups. Our study has demonstrated a negative appendectomy rate within the accepted range and was lower when compared to previously reported studies,

Females , older age patients and those with high preoperative WBC count exhibited a higher negative appendectomy rates. In order to prevent the oversight of any clinically significant and manageable conditions, it is imperative to incorporate a systematic histological investigation into the routine analysis of all appendicectomy specimens.

Ethical Approval:

All ethical issues were approved by the author. Data collection and patients enrollment were in accordance with Declaration of Helsinki of World Medical Association, 2013 for the ethical principles of researches involving human. Signed informed consent was obtained from each participant and data were kept confidentially.

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