PREVELENCE OF *HELICOBACTER* SPECIES IN SOME EDIBLE AND NON EDIBLE CHICKEN OFFALS.

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ABSTRACT

The current surveillance study was carried out to determine the prevalence of *Heticobacter* spp. in a total 120 samples of fresh chicken offals (30 from each of liver, gizzard, heart and cecal contents). Also it was undertaken to determine the effect of different methods of cooking on the survival of isolated organism. The samples were collected from different poultry slaughter and evisceration shops in Assiut city. The biotyping showed that 25 (83.3%), 28 (93.3%), 9 (30%) and 30 (100%) of liver, gizzard, heart and cecal contents samples respectively were positive for *Helicobacter* spp., and upon using polymerase chain reaction (PCR) assay only 9(30%), 9 (30%), 3 (10%) and 12 (40%) of these samples respectively were positive for the same organism. The positive samples of liver were found to be 3 (10%) *H.pylori* and 6 (20%) *H.pullorum*, while the positive samples of gizzard were found to be 3 (10%) for each of *H.pylori, H.pullorum* and *H.cinaedi*, as to heart samples appears to only be contaminated with *H.pullorum* with a percentage of 10%. Both *H.pylori* and *H.canis* could be isolated from 3 samples of cecal contents with a percentage of 10% for each, while *H.pullorum* could be isolated from 6 samples of cecal contents with a percentage of 20%. The highest isolation rate of *Helicobacters* was achieved with cecal contents followed by liver and gizzard samples (both have an equal percent of contamination) then heart samples. *H.pullorum* has the highest incidence of isolation from both liver and cecal contents. Different methods of cooking were applied on the liver and gizzard samples, the obtained results pointed out that *Helicabacter* spp. could be isolated from 22.2% of grilled liver samples and from 11.1% of boiled gizzard samples, while could not be detected from gizzard samples which exposed to boiling then frying. These results indicated meat thermometer is necessary to safe cooking and internal temperature of 82 ^{o}C is enough to eliminate the *Hilicobacter* hazard. When visual colour and doneness indicators replaces the thermometer, the combined effect of boiling and frying is the efficient method of cooking for the destruction of *Helicobacter* organism. The public health significance of the isolated microorganisms as well as the preventive measures were discussed.

Keywords: *Prevalence - Helicobacter species, PCR, chicken, offals, cooking, survival.*

INTRODUCTION

Chickens are economic source of animal protein, consumed on a large scale in Egypt. Also their edible offals are highly demanded due to their high biological value, reasonable price, agreeable taste and ease of serving. Poultry meat production depends heavily on live bird markets because of marketing infrastructure insufficiencies, and consumer factors. Over 70 percent of the broilers are sold at small retail poultry shops. There are around 15,892 such retail shops in Egypt that sell either live or freshly slaughtered birds to the consumer. In addition, 4,305 small slaughtering and de-feathering points exist that sell freshly slaughtered and chilled birds and bird parts and most of these slaughtering points are within residential areas, (FAO, 2006).

In small-scale slaughtering facilities, birds are slaughtered and then scalded in hot water. The carcasses are then plucked and eviscerated, mostly by hand. At evisceration, the vent is opened, the internal organs are removed, and the gizzard, liver and heart may be harvested. These edible organs can be contaminated through spillage of the contents of the intestines. After evisceration, they are often washed, which may contribute to the dissemination of bacteria on and among them. (Arnold, 2007).

The genus *Helicobacter* has expanded rapidly over the past decade and as more animal hosts are investigated, other new species will undoubtedly be discovered and the concept of *Helicobacter* will continue to expand. The genus *Helicobacter* nowadays includes at least 26 formally named species, with additional novel species in the process of being characterized (Whary and Fox, 2004).

Members of the genus *Helicobacter* are micro aerobic, spiral motile rod by flagella. The genus can roughly be divided into gastric and enterohepatic *Helicobacter* species. Both believed to be zoonotic pathogens (Josenhans *et al.,* 2000).

All gastric *Helicobacter* species have strong urease activity, they manage to survive gastric acidity by expressing urease at a level higher than that for any other known microorganism (Sach *et al.,* 2003), *Helicobacter pylori* is considered the most prevalent of this species. The bacteria may be transform into coccoid or spherical cell (Dewhirst *et al.,* 2000), the actual phenomenon of this transformation has been investigated in *H.pylori* but not in other *Helicobacter* spp., several factors may influence the spiral to coccoid conversion of *H.pylori*, such as acid pH, stress, oxygen, temperature and nutritional starvation.

In comparison to spiral forms, coccoid shapes of *H.pylori* have been demonstrated to possess damaged genomic DNA, less total amounts of DNA and RNA, a loss of membrane potential and considerably diminished levels of intracellular ATP, indicative of a metabolic state of cellular degeneration (Taneera *et al.,* 2002).

It was found that *H.pylori* causes recurrent abdominal pain (Ozen *et al.,* 2001), and growth retardation in children (Tasar *et al.,* 2006). Also there was a close relation between eradication of *H.pylori* and prevention of gastric cancer (cheung *et al.,* 2007).

In Egypt, the prevalence of *H.pylori* infection was reported to be 60% up to 86% of adult population (Frenck *et al.,* 2006). This prevalence is considered a public health problem especially among low socioeconomic level population, probably due to the condition that favor the acquisition of infection such as precarious hygiene, crowded living conditions and absence or deficiency of sanitation (Graham *et al.,* 1991).

The enterohepatic *Helicobacter* species have the characteristics of ultra structure and physiology in common with the gastric species.

To date, these enterohepatic agents have been identified in the intestinal tract and /or the liver of humans, mammals and birds (Inglis *et al.,* 2006). The most common members of this group are *H.pullorum*, *H.cinaedi* and *H.canis*. Various human cases of gastroenteritis, an increase of liver enzymes and gallbladder cancer were associated with *H.pullorum* infection (On *et al.,* 2002), it has also been suggested that *H.pullorum* plays a role in Crohn's disease (Bohr *et al.,* 2002).

H.cinaedi infection has been associated with septicaemia and meningitis in a neonate (Orlicek *et al.,* 1993), also it play an important role in the pathophysiology of cholesterol gallstone development in mice and possibly in humans (Maurer *et al.,* 2005). Recently, the DNA of *H.cinaedi* was detected in patients with pancreatic exocrine cancer (Nilsson *et al.,* 2006). Both *H.pullorum* and *H.canis* are included in the causes of intestinal and hepatobiliary disease in birds (Solnick and Schauer, 2001).

To date various Gram-negative bacteria species including *H. pulorum* have been shown to produce CDT "Cytolethal distending toxin" (Taylor *et al.,* 2003). This toxin blocks cell proliferation by activating DNA damage induced cell cycle checkpoint response (Li *et al.,* 2002).

Helicobacter pylori was found to resist cold starvation for an extended period of time (Nilsson *et al.,* 2002), also packaging in vacuum or air had little effect on survival of the organism, Stevenson *et al. (*2000). Heat is the most common, inexpensive, simplest and effective method used to destroy microorganisms, that it denatures the proteins and enzymes of the microorganisms. The meat thermometer is important tool to make sure all potentially harmful bacteria have been destroyed through proper cooking temperature. Hence, the United States Dept. of Agriculture (USDA) recommends consumers use a food thermometer to ensure the internal temperature of chicken breasts and whole chicken reaches 76.7˚C and 82˚C, respectively (USDA, 2005).

It is worth mentioning that most of the Egyptian kitchens do not use a meat thermometer. They determine the doneness of meat most often by cutting the meat to evaluate changes in color and texture, or by other subjective techniques. In contrast, the food safety messages promoted to the U.S. consumer have changed from judging the doneness of cooked meat on the basis of its appearance to using a thermometer. This was in response to increasing evidence that the visual appearance of cooked meats does not necessarily indicate that a microbiologically safe cooking temperature has been achieved (Lyon *et al.,* 2000). Also it is cleared that improper cooking is one of the main factors causing food borne illness (Panisello *et al.,* 2000).

The aim of this study was to investigate the occurrence of *Helicobacter* spp. in chicken offals,

collected from different poultry slaughter and evisceration shops in Assiut City employing combination of a modified conventional culture, Biochemical identification and a PCR assay. Also it was undertaken to determine the effect of different methods of cooking on the survival of isolated organism.

MATERIALS and METHODS

Collection of samples:
One hundred and twenty samples of fresh chicken (gizzard, heart, liver and cecum), 30 samples each were collected from different poultry slaughter and evisceration shops in Assiut city. Samples were collected carefully avoiding cross-contamination. The samples of each bird (liver, heart gizzard as well the cecum) were obtained immediately after slaughter. Each sample was packed separately into a sterile plastic bag using disposable gloves, tightly closed and placed in a cool box packed with ice during transportation to the laboratory and kept cool until microbiological examination within two hours.

Microbiological analysis for isolation of *Helicobacter* spp.:
Isolation procedure:
Isolation of *Helicobacter* species was adopted as described by Stevenson *et al.* (2000). From each of gizzard, heart and liver, approximately 2 g sample was aseptically weighed into separate sterile 9 mL Helicobacter Special Peptone Broth (HPSPB) tubes supplemented with (Helicobacter antibiotic supplement (SIGMA) and 5% calf serum). The tubes containing the samples were homogenized using a vortex then incubated at 37°C for 48 hours under microaerophilic condition using an anaerobic jar and gas generating kits (Oxoid CN 35).

From each enrichment culture, a loopful was streaked onto Columbia Blood Agar (CBA) plate supplemented with (*Helicobacter* antibiotic supplement and 5% dfibrinated sheep blood) and incubated at 37°C for 4 days under microaerophilic condition.

The cecal contents was inoculated by passive filtration method as recommended by Steele and McDermott (1984). For passive filtration, 5 grams of cecal contents were mixed with sterile phosphate buffer saline (approximately 1/10 dilution) to produce a suspension. Approximately 100 µl of this suspension was then carefully layered onto a 0.65 µm filter, which was previously placed on top of Columbia blood agar plate with supplements. The bacteria are allowed to migrate through the filter for 45 minutes at 37°C. The filter was then removed, the fluid that had passed through the filter was spread with a sterile glass, and the plates were incubated at 37°C for 4 days under microaerophilic condition.

Suspected colonies of *Helicobacter* spp. (grayish white) were checked by Gram's stain. Gram - negative, gently curved, slender rod bacteria were subjected to oxidase, catalase and microscopic examination of wet mounts under phase contrast. Colonies giving reactions typical for *Helicobacter* spp. were purified by subculturing on blood agar plates. *Helicobacter* spp. were confirmed using the protocol recommended by Zenner (1999) and Murray *et al.* (2003). As phenotypic and biochemical tests may result in misidentification thus confirmation of the suspected isolates was carried out using PCR.

***Helicobacter* genus-specific polymerase chain reaction (PCR):**
Application of PCR for identification of 16S rRNA gene of *Helicobacter* species was performed essentially by using Primers (Pharmacia Biotech) as shown in the following table:

Table 1: Primer sequences for *Helicobacter* spp. polymerase chain reaction

Target gene	Oligonucleotide sequence (5′ → 3′)	Amplicon length (bp)	Reference
16S rRNA (F)	5′-CTATGACGGGTATCCGGC-3′	375	Moyaert *et al.* (2008)
16S rRNA (R)	5′-ATTCCACCTACCTCTCCCA-3′		

DNA preparation from bacterial culture: (Riley *et al.*, 1996)

Helicobacteria were harvested from their specific agar plates by suspending one or two colonies in 20 ml phosphate buffered saline (pH 7.4) and the suspension was then heated at 100ºC for 20 minutes. From this suspension, a 5 µl aliquot was directly used as a template for PCR amplification.

DNA amplification: (Germani *et al.*, 1997):

The amplification was performed on a Thermal Cycler (Master cycler, Eppendorf, Hamburg,

Germany) using 25 µl of PCR mixture containing 3 µl of boiled cell lysate, 250 µM of each desoxynucleotide triphosphate, 1.5 U of Taq DNA polymerase (Biotools, Madrid, Spain), buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl and 3 mM MgCl2, Biotools), 1 µM of the primers mecA-R, mecA-F; 0.8 µM of icaA-R, icaA-F and 0.8 µM of icaD-R, icaD-F. Amplification conditions included 35 cycles consisting of denaturation (94°C for 2 seconds), primer annealing (53°C for 2 seconds) followed by for final extension (72°C for 30 seconds). Amplified products were analyzed by 2% of agarose gel electrophoresis stained with ethidium bromide and visualized and captured on UV transilluminator.

Effect of cooking on the survival of *Helicobacter* spp:

Preparation of samples:

The same samples of gizzard and liver used in the survey study were used in the cooking experiment. Cooking was done as rapid as possible after sampling as raw.

Cooking methods: Pourkhalili *et al.* (2013)

Liver samples were subjected to grilling using burning stove where the sample were placed at 10 cm above the flame for 10 min with turning by 2 minutes interval (developing internal pink color). The internal temperatures just after grilling was 70°C as determined using digital thermometer (thermometer ST-131 waterproof digital). Grilled samples were bacteriologically examined when their temperatures reached 15 °C.

The gizzard samples were subjected to two methods of cooking, the 1st was boiling while the 2nd was boiling then frying. Boiling of gizzard samples was performed at approximately 97°C (water temperature) for 30 min (developing internal grayish brown color and the juices run clear) in stainless steel pan. The internal temperature immediately after cooking was 78°C. The boiled samples were bacteriologically examined when their temperatures reached 15 °C then immediately fried in a pan using sunflower oil for 20 minutes (developing golden colour). The internal temperature immediately after frying was 85°C. Also the fried samples were bacteriologically examined when their temperatures reached 15 °C. The bacteriological examination of both control and cooked samples was carried out as described before according to Zenner (1999), Stevenson *et al.* (2000) and (Murray *et al.,* 2003).

RESULTS

Table 2: Prevalence of *Helicobacter* spp. in edible and non edible offals.

Type of sample	No. examined samples	***Helicobacter* spp.**				**Classified *Helicobacter* spp.**							
		Biochemical		PCR		*H.pylori*		*H.pullorum*		*H.canis*		*H.cinaedi*	
		No. of +ve	%	No. of +ve	%	No. of +ve	%	No. of +ve	%	No. of +ve	%	No. of +ve	%
Liver	30	25	83.3	9	30	3	10	6	20	0	0	0	0
Gizzard	30	28	93.3	9	30	3	10	3	10	0	0	3	10
Heart	30	9	30	3	10	0	0	3	10	0	0	0	0
cecum	30	30	100	12	40	3	10	6	20	3	10	0	0
Total	120	92	76.7	33	27.5	9	7.5	18	15	3	2.5	3	2.5

Table 3: Effect of grilling on the survival of *Helicobacter* spp. in liver samples.

Type of samples	No. of examined samples	+ve samples of *Helicobacter* spp.	
		No	%
Raw liver (control)	9	9	100
Grilled liver	9	2	22.2

Table 4: Effect of boiling and both boiling and frying on the survival of *Helicobacter* spp. in gizzard samples

Type of samples	No. of examined samples	+ve samples of *Helicobacter* spp.	
		No	%
Raw gizzard (control)	9	9	100
Boiled gizzard	9	1	11.1
Boiled and fried gizzard	9	0	0

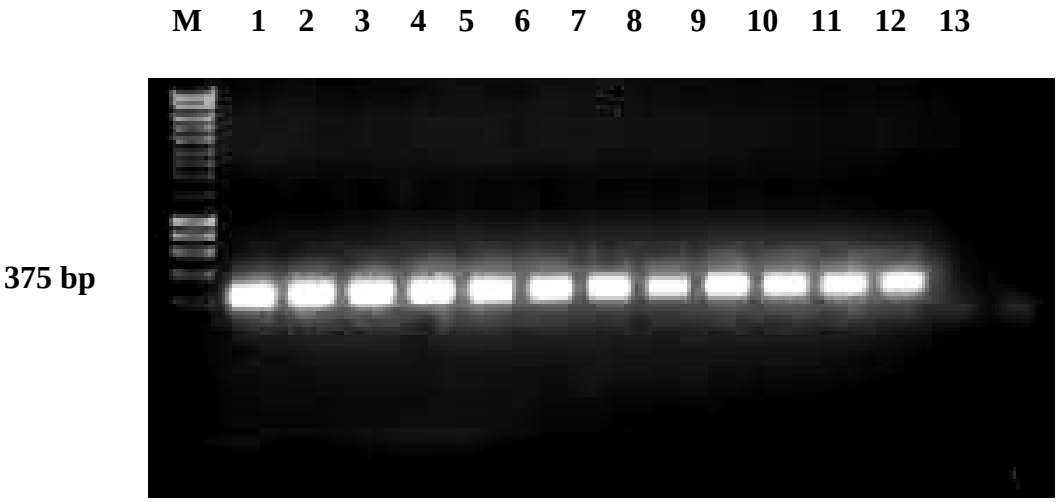

Fig. 1: Agarose gel electrophoresis of PCR amplification products using genus specific primer for detection of *Helicobacter* species.

Lane M: 100 bp ladder as molecular DNA marker.
Lane 1: Control positive for *Helicobacter* species.
Lane 13: Control negative for *Helicobacter* species.
Lane 2 to Lane 12: Positive strains *as* Helicobacters.

DISCUSSION

Helicobacter is a significant problem within the developing world, and its importance is unlikely to diminish in the foreseeable future, which abound the opportunities of future research. In this study, samples of cecal contents of chicken were investigated as a source of contamination with *Helicobacter*. The extent of contamination of gizzards, livers and hearts with *Helicobacter* was also studied.

Helicobacter species are fastidious bacterial pathogens that are difficult to isolate, therefore using PCR method is more helpful for detection of these fastidious bacteria.

Table (1) shows that thirty samples from each of liver, gizzard, heart and cecal contents of chicken were examined for the presence of *Helicobacter*s.

Biochemical tests showed that 25 (83.3%), 28 (93.3%), 9(30%) and 30 (100%) of these samples respectively were positive for this organism, and upon using PCR assay only 9 (30%), 9(30%), 3(10%) and 12 (40%) of these samples, respectively were positive for the same organism.

The positive samples of liver were found to be 3(10%) *H.pylori* and 6 (20%) *H.pullorum*, while the positive samples of gizzard were found to be 3 (10%) for each of *H.pylori, H.pullorum* and *H.cinaedi.* Also the same table shows that heart samples only contaminated with *H.pullorum* with a percentage of 10%. Both *H.pylori* and *H.canis* could be isolated from 3 samples of cecal contents with a percentage of 10% for each, while *H.pullorum* could be isolated from 6 samples of cecal contents with a percentage of 20%.

The highest isolation rate of *Helicobacter*s was achieved with cecal contents followed by liver and gizzard samples (both have an equal percent of contamination) then heart samples. *H.pullorum* has the highest incidence of isolation from both liver and cecal contents samples.

Regarding the highest isolation rate from cecal contents, (celeen *et al.,* 2006) assumed that the lower segments of the intestinal tract are the predominant colonization sites for *H.pullorum* in broiler chickens. *H.pullorum* may gain access to the liver by retrograde movement from the duodenum. Alternatively, it may transfer from the gut lumen to the portal circulation.

A number of factors have been reported in the colonization of the organism in chicken, among these risk factors are inadequately cleaned and disinfected houses and facilities, presence of pests and pets, contaminated water supplies as well as boots and clothes of farm worker (Newell and Fearnley, 2003).

Several reports were published about the incidence and prevalence of *H.pullorum* from cecal samples. Zanoni *et al.* (2007), Mohamed, (2011), Beren, (2013) and Jamshidi *et al.* (2014), found an incidence rate of 100%, 50% 32.29% and 41% respectively upon sampling the caecal contents of chicken, which are higher than that obtained in our result.

Regarding to isolation rate of *H.pullorum* in liver samples (Ceelen *et al.,* 2006; Liesbeth *et al.,* 2006; Moemen *et al.,* 2010; Mohamed, 2011 and Hassan *et al.,* 2014), found this rate to be 4.6%, 4.6%, 47%, 14% and 47% respectively, the present study declared higher incidence than that recorded by (Ceelen *et al.,* 2006), (liesbeth *et al.,* 2006) and (Mohamed, 2011), on the other hand (Moemen *et al*., 2010) and (Hassan *et al.,* 2014) recorded higher results than that obtained in our study.

Also (Mohamed, 2011) could detect *H.pullorum* in 6% of gizzard samples, he reported that *H.pylori* was found in 16%, 4% and 6%, of intestinal contents, liver and gizzard, respectively. He also announced that *H.canis* could be isolated from 2% of both intestinal contents and gizzared samples, while *H.cinaedi* could be isolated only from 6% of intestinal contents.

Cooking is the process of producing safe and edible foods. It is clear that cooking has been around for a long time and continues today to play a fundamental role in daily life across the globe. Cooking was first used for preservation but it has evolved and now it is a form of entertainment and creativity for many people. The fundamental types of cooking are boiling, grilling and frying. Boiling is a method of cooking in which food cooked in boiling water for a certain time according to the type of food, grilling is cooking of food using a direct dry heat, while frying is the cooking of food in oil or fat. Common types of food that are grilled and fried include fish, meat and chicken (EUFIC, 2010).

To explore the efficiency of Egyptian cooking methods in elimination of *Helicobacter* contaminating chicken offals, gizzard and liver samples were cooked in ways similar to what is done at home kitchen. From the tabulated data in table (3), the percentage of *Helicobacter* spp. in grilled liver samples was 22.2%, and as presented in table (4) the same organism was recovered from 11.1% of boiled gizzard samples and could not be detected from gizzard samples which exposed to boiling then frying.

The consumer opinion that judging offals doneness using the appearance, resulted in elimination of *Helicobacter* only by the combination effect of both boiling and frying. The internal temperature of the treated samples (82 ºC) exceeded the safety cooking temperature (76.4 ºC) recommended by (USDA, 2005).

The survival of *Helicobacter* spp. in some cooked samples may be attributed to the fact that, consumers considered the appearance of doneness signs is the end point of cooking (the minim cooking temperature achieved but not continue for proper time). In this respect Whyte *et al.* (2006) mentioned that chicken liver must cooked to internal temperature in excess of 70 ºC and held at that temperature for 2 to 3 minutes.

In conclusion *Helicobater* organism was detected in chicken offals which may contaminate the carcasses, that all present a potential hazard for public health. Therefore, it is essential to identify the risk factors for

the presence of *Helicobacters* for human health, and proper cooking for both chicken carcasses and their offals. Meat thermometer is necessary to safe cooking and internal temperature of 82 ºC is enough to eliminate the *Hilicobacter* hazard. When visual colour and doneness indicators replace the thermometer, our results indicated that the combined effect of boiling and frying is the best method of cooking for the destruction of *Helicobacter* organism.

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مدي تواجد ميكروبات الهليكوباكتر في بعض احشاء الدجاج الصالحة والغير صالحة للإستهلاك

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معاينة البحث نفّذت لتعيين مدى انتشار طائفة الهليكوباكتر في ١٢٠ عينة طازجة (٣٠ عينة من كل من الكبد والقونصة والقلب ومحتويات المعي الأعور) من ذبائح الدجاج وأيضاً تولي هذا البحث تعيين تأثير الطرق المختلفة من الطهي على تواجد الميكروب في الأحشاء الصالحة للإستهلاك. جمعت العينات من محلات مختلفة لذبح وتنظيف الطيور بمدينة أسيوط. أظهرت نتائج الأختبارات البيوكيميائية أن ٢٥ (٨٣.٣%)، ٢٨ (٩٣.٣%)، ٩(٣٠%)، ٣٠(١٠٠%) من عينات الكبد والقونصة والقلب ومحتويات الأمعاء على التوالي أنها إيجابية لطائفة الهليكوباكتر. بعد إجراء اختبار تفاعل البلمرة المتسلسل وجد أنه ٩ (٣٠%)، ٩ (٣٠%)، ٣ (١٠%)، ١٢(٤٠%) من هذه العينات على التوالي هي فقط الإيجابية لنفس الميكروب. وكانت عينات الكبد الإيجابية عبارة عن ٣ (١٠%) من الهليكوباكتر بيلوري و ٦(٢٠%) من الهليكوباكتر بلورم بينما كانت العينات الإيجابية للقونصة عبارة عن ٣ (١٠%) لكل من الهليكوباكتر بيلوري والهليكوباكتر بلورم والهليكوباكتر سينادي أما بخصوص عينات القلب فكانت ملوثة فقط بالهليكوباكتر بلورم بنسبة ١٠%. وكلاً من الهليكوباكتر بيلوري والهليكوباكتر كانيز عزلت من ثلاث عينات من محتويات المعي الأعور بنسبة ١٠% لكلاً منهما، بينما عزلت الهليكوباكتر بلورم من ٦ عينات من محتويات الأمعاء بنسبة ٢٠%. وكانت أعلى نسبة عزل لميكروب الهليكوباكتر من محتويات المعي الأعور يليها عينات الكبد والقونصة حيث أنهما متساويان في نسبة التلوث وبعد ذلك تليهم عينات القلب. وكان لميكروب الهليكوباكتر بلورم أعلى نسبة عزل من عينات الكبد ومحتويات المعي الأعور. ولقد أجريت عدة طرق للطهي على عينات الكبد والقونصة حيث أشارت النتائج إلى أن طائفة الهليكوباكتر تم عزلها من ٢٢.٢% من عينات الكبد المشوية ومن ١١.١% من عينات القوانص المغلية ولم يتم عزل الميكروب من عينات القوانص التي تم غليها ثم تحميرها. ولقد دلت هذه النتائج على ضرورة استخدام الترمومتر لتحقيق الطهي الآمن وأن الطهي لدرجة ٨٢ درجة مئوية داخل الكبد والقونصة كافي لقتل الهيليكوباكتر وفي حالة عدم التمكن من إستخدام الترمومتر في عملية الطهي وإستبدالهم بالتقييم الحسي للنضج فإن أفضل طرق الطهي لقتل الميكروب هي الغلي مصحوباً بالتحمير.