Assiut Provincial Laboratory,
Animal Health Research Institute.

# THE RELATIONSHIP BETWEEN FEEDING ON FISH MEAL AND *Aeromonas hydrophila* INFECTION IN BROILER CHICKENS IN ASSIUT GOVERNORATE

(With 5 Tables and 4 Figures)

By

***FATMA M. MOHAMED and M.A. MOHAMED****

* Dept. of Poultry Dis., Col. Vet. Med., Assiut Univ., Assiut-Egypt.
*Corresponding author: moemenassiut@hotmail.com

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## العلاقة بين التغذية علي عليقة مسحوق السمك والاصابة بميكروب الايروموناس هيدروفيلا في بداري الدجاج بمحافظة أسيوط

***فاطمة مختار محمد ، مؤمن عبد العظيم محمد***

هناك بعض الدراسات القليلة والتي تم فيها وصف ميكروب الايروموناس في دجاج التسمين ولذلك تم في هذه الدراسة عمل فحص حقلي لالقاء الضوء علي مدي انتشار ميكروب الايروموناس في الدجاج المغذي علي علائق السمك وذلك من خلال ١٣٧ عينة اجمالية منها ٣٧ من مسحوق السمك و٥٠ عينة مسحات شرجية من دجاج مصاب و٥٠ من دجاج سليم ظاهريا. هذا وقد تم التعرف على الايروموناس هيدروفيلا مورفولوجيا وبيوكيميائيا وتأكد العزل في ٣٤ من ١٣٧ عينة اجمالية بنسبة ٢٤,٨% وكانت أعلي نسبة لوجود الميكروب في علف السمك بنسبة ٤٨% يليها ١٨% في عينات مسحات المجمع ثم ١٤% في العينات المأخوذة من دجاج سليم ظاهريا. ولاظهار العلاقة بين العترة المعزولة من العينات المختلفه تم توصيف المحتوى البلازميدى لـ٢٦ عترة معزولة من الايروموناس هيدروفيلا (١٠عينات من مسحوق السمك و ١٦ عينة من الدجاج المريض (٩) والدجاج السليم (٧). هذا واظهر الفحص وجود ١٤ عترة مختبرة من أصل ٢٦ عينة معزولة بنسبة (٥٣,٨%) الوزن الجزيئي لهم (٢,٧-٥٧ كيلو بايت) وقد تم فصل نوعين شائعين من عينة السمك ومن الدجاج المصاب والسليم مما برهن بالتأكيد علي اصابة الدجاج بنفس المعزوله الموجودة في علف السمك. مع العلم أن الايروموناس هيدروفيلا المعزولة من الدجاج المصاب والسليم أكثر ضراوة من العترات المعزولة من علف السمك في كلا النسختين. ولتوضيح ميكانيكية الضراوة للعترات المعزولة تم اختبار تواجد العوامل التالية (تحلل الدم، جين ايروليسين (*aerA*) وجين الحقن من النوع الثالث (*ascV*) وبلازميد الضراوة) بين العترات المعزولة. لقد لوحظ أحتواء العزلات المختبرة على هذه العوامل مع زيادة واضحة لها فى العترات المعزولة من الحالات المرضية عنه فى العترات المعزولة من مسحوق السمك. ولاثبات اثر امتلاك عوامل الضراوة على الميكروبات المعزولة تم حقن كتاكيت دجاج عمر يوم تحت الجلد وبعد الحقن ب٤٨ ساعة

لوحظ ابتداء نفوق الكتاكيت بمجموع كلى للنفوق ٨٠% فى الكتاكيت المعدية بالعترة الضارية عنه فى العترة الغير ضارية ٥٠%. وأثبت الفحص التشريحي وجود احتقان في الكبد وعدم امتصاص لكيس المح بشكل واضح. كما تم تحديد مستويات المقاومة لعشرة من المضادات الحيوية عاى ٢٦ عترة موزعة علي مجموعتين الاولي معزولة من الدجاج والثانية معزولة من العلف. وجد أن معظم العترات المعزولة من الدجاج كانت مقاومة للأمبيسيلين والأموكسيسيلين والأريثروميسين ومقاوم جدا للتتراسيكلين، وحساسة لدرجة كبيرة للجنتاميسين وسيبروفلوكساسين بينما العينات المعزولة من علائق السمك كانت ١٠٠% مقاومة للاموكسيسيللين والأريثرومايسين وهذه النتائج توضح اختلاف المضادات الحيوية باختلاف مصدر العينة حيث أن الميكروب المعزول من دجاج التسمين أكثر مقاومة للمضادات الحيوية من الميكروب المعزول من العلف نتيجة الاستخدام الزائد للمضادات الحيوية فى مزارع الدواجن. وعلية فان هذه النتائج تدل علي ضرورة القضاء علي الميكروبات الملوثة لعلائق السمك قبل اضافتها علي العليقة المقدمة لدجاج التسمين لتقليل نسبة الأصابة بميكروب الايروموناس هيدروفيلا في دجاج التسمين.

## SUMMARY

A total of 137 samples including fish meals (37samples) and cloacal swabs from diarrheic broiler chickens (50 samples) as well as apparently normal chickens (50 samples) fed on ration supplemented with fish meal, were investigated for the presence of *A. hydrophila*. The overall prevalence level was 24.8% (34/137). The highest isolation rate was recorded from fish meal samples with a percentage 48.6% (18/37), followed by 18% (9/50) from diarrheic cloacal samples, whereas the lowest prevalence rate 14% (7/50) has been recorded from cloacal swabs of clinical healthy broiler chickens. Based on plasmid profiling, the relationship between 26 examined strains of *A. hydrophila* revealed 14 strains (53.8%) having plasmids with a molecular weight ranged from 2.7kb- 56kb. Two common clones found between strains of fish meal and broiler chickens isolates, that provides a suggestive evidence of successful colonization and infection by particular strains of certain *A. hydrophila* after transmission from fish meal to broiler chickens. Pathogenicity of *A. hydrophila* has been associated with the virulence factors such as hemolysin production, virulence genes (*aerA* and *ascV*). The detection of the presence of such virulence factors is a better indicator of the potential risk for their pathogenicity. Virulence factors were detected among examined strains but the higher frequency of possession was observed in diseased birds than that mentioned from strains recovered from fish meal and clinically healthy birds. Pathogenic potential and its relation to virulent factors were investigated in one-day-chicks infected via subcutaneous (*S/C*) route. Group infected with

virulent *A. hydrophila* strain that having all examined virulent traits showed 80% mortality, in contrast to 50% mentioned with avirulent strain within 48 hours post inoculation. Antibiotic susceptibility testing revealed 100% of clinical strains were resistant to ampicillin, amoxicillin and erythromycin and high resistance level to tetracycline while the fish meal strains presented 100% resistance to amoxicillin and erythromycin. Resistance among the *A. hydrophila* isolated from broiler chickens was higher than that observed with fish meal recovered strains. Eleven out of 26 (42.3%) examined isolates showing multiple drug resistance to the tested antibiotics, that is may indicate the use of these antimicrobials in commercial farms and this can favor the development of resistant bacterial strains. The present work highlights an important incidence of *A.hydrophila* and its possession to virulence genes as well as animicrobial resistance that reinforced their potential pathogenicity to infected chiks and its possibility to causing illness and losses in broiler chickens. Hence, continuous monitoring of fish meal is important to identify potential pathogenic *A. hydrophila* before its addition to food ration to reduce the risk of infection.

***Keywords:*** *A. hydrophila, fish meal, broiler chickens.*

# INTRODUCTION

The production of good quality poultry meat is of the utmost importance in poultry farms worldwide. Rations can affect the success of the poultry business, due to production costs incurred 60-70%. Increased production costs can be overcome by finding a quality feed ingredients. One of the feed ingredients of high nutrient value that deserves attention is fishmeal due to the well-balanced amino acid profile that will supplement any deficiency of the amino acids in vegetable protein such as soyabean (Miles and Jacobs, 1997). Also fish meals contain omega −3 and −6 fatty acids that protect health of chicks and reduce their dependence on antibiotics and other drugs (Miles and Chapman 2006).

Concern over bacterial infection and contamination in poultry environment has become an issue of great importance. Fish meal has been suggested as a source of dissemination of *Aeromonads* to the birds (Karimi, 2006).

*Aeromonas* is an opportunistic pathogen of a variety of aquatic, terrestrial animals and birds. *Aeromonas* is oxidase-negative and catalase

positive, glucose fermenting, facultative anaerobic, Gram-negative, rod-shaped, motile and non-motile bacteria (Martin-Carnahan and Joseph, 2005). *Aeromonas hydrophila* have received increasing attention as opportunistic as well as primary pathogens in humans, aquatic and animals. Human infections include gastrointestinal tract syndromes, and extraintestinal symptoms such as; septicemia, wound infections, meningitis, endocarditis and osteomyelitis (Janda and Abbott, 2010).

The studies related with the motile aeromonads of poultry are limited. *A. hydrophila,* either alone or in combination with other organisms, can cause localized and systemic infection in avian species including poultry (Glunder and Siegmann 1989; Shane and Gifford, 1985). *Aeromonas* was recorded in chickens and turkeys suffering from enteritis and watery feces (Efuntoye, 1995) as well as in ducks suffering from salpingitis, septicemia and/ or air saculitis (Bisgaard *et al.,* 1995; Li, *et al.,* 1998). *A. hydrophila* was isolated from different ages of dead or sacrificed chickens, ducks and turkeys with percentages of 15, 22.5 and 20% respectively by Amal (2007) in Upper Egypt.

Virulence determinants are responsible for the establishment and maintenance of an infection in the host, including cell structural lipopolysaccharides (LPS), outer-membrane proteins (OMPs), pili and flagella, a type III secretion system (T3SS) acting as adhesion structures, and extracellular factors such as enzymes and toxins (Janda and Abbott, 2010). Among the exotoxins, aerolysin (Heuzenroeder *et al.,* 1999), heat-stable cytotonic enterotoxin, heat-labile cytotonic enterotoxin (Albert *et al.,* 2000) and cytolitic enterotoxin (Xu *et al.,* 1998) seem to play an important role in pathogenesis. As well as plasmids are well known carriers of several virulence determinants that plays an important role in the pathogenesis of *Aeromonas*. Those plasmids confer resistance to antimicrobials which can be transferred along with transfer of these plasmids (Casas *et al.,* 2005). The type III secretory system (TTSS) that has been reported in several *Aeromonas* isolates has recently been shown to be plasmid encoded in *A. salmonicida* and *A. hydrophila* (Ebanks *et al.,* 2006).

One of the most important problems facing treatment of bacterial infections is the emergence of bacterial isolates that are resistant to an antimicrobial agent and represents a continuing ecological battle to achieve a natural host-parasite balance. This shift in susceptibility greatly affects our ability to successfully treat empirically. So, periodic monitoring of drug resistance of these organisms should be carried out in

different geographical areas so that appropriate agent can be chosen for empiric therapy.

Increased awareness of *Aeromonas* species in animals and human has stimulated interest about possible existence and distribution among chickens in Assiut. This study was designed for carrying out the following aims. The first was to determine the prevalence of *A. hydrophila* among fish meal and broiler chicken samples. The second aim was to establish the relationship of strains from fish meal specimens and from broiler chickens by using plasmid profiling. The third aim to screen the presence of virulence-associated genes in isolates from fish meal samples and diseased diarrheic and apparently healthy broiler chickens.

## MATERIALS and METHODS

### I- Recovery of *Aeromonas* from the examined samples

Thirty seven samples of white fish meal collected from different companies for ration manufacturing as well as from several broiler farms composed its ration manually. Twenty five grams of respective samples were homogenized in 225 ml of alkaline peptone water (5%) pH 8.6. 0.1ml amounts were plated on *Aeromonas* medium (HiMedia Laboratories, India) containing 5 mg/liter of ampicillin was used as selective isolation medium for *Aeromonas* spp. The agar plates were incubated at 30°C for 24 h.

Fifty samples of cloacal swabs were collected from diseased birds suffered from diarrhea (watery mucoid) as well as 50 cloacal swabs from apparently healthy birds were swabbed and fed on ration contained fish meal as concentrates and as replacement to part of soybean used. All swabs were taken into sterile containers and examined within two hours of sampling. As mentioned before, samples incubated firstly in alkaline peptone water containing ampicillin for 24 hours at 30ºC to decrease the chances of growth of other enteric bacteria

The suspected colonies were spread on 5% sheep blood agar with ampicillin, to examine the hemolytic phenotype of the genus *Aeromonas.* The isolates were confirmed through oxidase and catalase tests, and also found to be resistant to ampicillin and Voges–Proskauer (VP) positive. For species identification, each strain identified as *A.hydrophila* was examined using a battery of biochemical tests (hydrogen sulphide formation, glucose and lactose fermentation, gas from glucose, acid formation from sucrose and inositol, indole test,

citrate as carbon source, arginine hydrolysis and urease test) as described by (Beaz-Hidalgo *et al.,* 2010).

**II- Plasmid profiles for assessment of homology between the isolated strains**

i- *Preparation of bacteria*: One single colony-forming unit (CFU) of *Aeromonas hydrophila* was suspended in 5 ml brain heart infusion broth (Oxoid Ltd., Hampshire, UK) and incubated overnight at 30°C in shaking water bath, shaking at 50 revolutions per minute (RPM). One ml of the overnight culture was pelleted in a centrifuge at 3000x g for 10min at 4°C.

**ii-** *Plasmid isolation*: Plasmid DNA isolated from 26 *A. hydrophila* strain using Qiagen plasmid isolation kit (Qiagen Inc, USA) and electrophoresed on 0.8% agarose gel in the presence of molecular weight markers (λ DNA *Hind-III*/*EcoRI*, Gibco).

**III- Prevalence of virulence traits**

- Twenty six *A. hydrophila* (ten from fish meal samples, 9 from diseased birds and 7 from clinically healthy birds) were examined for the prevalence of virulence traits (Hemolysin, *aerA* gene as well as virulence plasmid).

**- Hemolysin detection.** The production of hemolysin was assayed by recording the lysis of sheep erythrocytes in agar plate (5% sheep blood in brain heart infusion agar) was recorded as clearance of the medium around the growth after 24 h at 25°C (Wong *et al.,* 1998).

**- Detection of aerolysin (*aerA*) and a gene encoding an inner membrane component of the TTSS (*ascV*) genes:**

**- Bacterial DNA extraction for PCR.** Approximately 100 ml of a Tryptone soya broth (Oxoid) culture grown for 16 h at 30°C was used for DNA extraction using the Wizard Genomic DNA extraction kit (Promega Madison, USA) in accordance with the manufacturer's instructions. Subsequently, 5 ul of the DNA solution was used as a template for PCR amplification.

**- PCR amplification.** PCR was performed using 4pairs of primers A1( 5'-ATG ACC CAG TCC TGG CAC GG–3'), A2 ( 5'-GCC GCT CAG GGC GAA GCC GC-3') and Oligonucleotide primers (ASCV-fwd [5-ATG GAC GGC GCC ATG AAG TT-3 and ASCV-rev 5-TAT TCG CCT TCA CCC ATC CC-3) were designed to amplify a 480-bp aerolysin (*aerA)*and 710-bp *ascV* gene respectively. (Kannan *et al.*, 2001; Burr *et al.,* 2002). PCR assay was performed using a 1X master mix PCR reaction in a total volume of 50 µl 25µl of Go *taq* green master

mix (Promega, madison, USA), 20 pmoles each of forward and reverse primer (1µl) for each gene and 5 µl of the extracted DNA and 16 µl of sterile double distilled water, to make the final volume of 50 µl. The amplification programme consisted of 35 cycles of 30 seconds at 94ºC, annealing for 30 seconds at 55ºC for and a 1-minute extension at 72ºC with a final extension at 72ºC for ten minutes in a Techne Cyclogene Cycler. The PCR products were separated in a 1% agarose gel. Electrophoresis was performed in 0.5 X Tris-borate-EDTA buffer for 1h at 100V. Amplicons were visualized with UV light after the agarose gel had been soaked in ethidium bromide solution (0.5 µg/ml) for 15 min.

**IV- Role of virulent traits possession in the pathogenicty of *A. hydrophila* to chickens:**

- A total of 40 one-day-old chicks (Ross) were obtained from a lot of chicks was delivered to commercial farm in Assiut. Water and commercial unmedicated balanced ration were given *ad libitum*. Ten chicks were sacrificed and subjected for laboratory examination to be sure that they were free from *A. hydrophila* infection.
- Two isolates were used for experimental study, one has all examined virulent traits and the second considered avirulent that haven't any of the examined virulent traits as well as isolated from clinically healthy birds.
- Each isolate was inoculated *SlC* in a group of 10 two-days-old broiler chicks with a dose 0.1-ml suspension of 1.5 X $10^9$ organisms (Shane and Gifford, 1985). Ten uninoculated chicks served as non-infected control.
- All chicks were kept for three weeks. All birds were examined daily for clinical signs; autopsies were performed on moribund chicks and subjected to bacterial examination.

**V- Antibiotic susceptibility testing:**

- Twenty six *A. hydrophila* isolated strains (10 from fish meal and 16 from clinical strains isolated from chickens) were tested for antibiogram using disk diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2005).
- Antibiotic disks were applied on the streaked cultures using Muller Hinton agar (Cat. No. M1084, HiMedia, India) with a Dispens-O-Disc dispenser (Difco Laboratories, Detroit, MI). According to the interpretation table of the Becton Dickinson Microbiology Company of USA, the zone diameter for individual antimicrobial agents was translated into sensitive, intermediate and resistant categories.
- The following antimicrobial disks were used: ampicillin 10ug, erythromycin 15 ug, amoxicilin (25ug), ciprofloxacin 5 ug, cefotaxime

(10 ug), doxycycline (30), tetracycline (30 ug), gentamicin 10 ug, neomycin 30 ug, colistin (25ug).

- Multiple Antibiotic Resistance (MAR) Indexing. The MAR index, when applied to a single isolate is defined as a/b, where a represents the number of antibiotics to which the isolate was resistant and b represents the number of antibiotics to which the isolate was exposed (Krumperman, 1985)

# RESULTS

**I - Prevalence of the *Aeromonas hydrophila***

- Of the total 137 samples that were tested by culture methods, 34 (24.8%) showed the presence of *Aeromonas hydrophila* Table 2. *Aeromonas hydrohila* was isolated from 18 (48.6%) of the 37 fish meal samples analysed. Of the 50 samples that were examined from diarrheic birds, *A. hydrophila* were recovered from 9 samples with a percentage 18%. In contrast to 7 samples were positive to *A. hydrophila* out of 50 cloacal swabs from clinically apparently healthy with a percentage 14%, the results are presented in Table 3.

**II- Plasmid profiles detection**

- Attempts were made to isolate plasmids from the 26 strains of *Aeromonas hydrophila*. Fourteen out of 26 examined isolates (53.8%) carried detectable plasmid DNA as shown in fig 1. Plasmid profile analyses revealed that all plasmid-containing strains have multiple plasmids from 3 to 6 in number that ranging in size from 2.7-56 Kb. (Fig.1). There are two common clones between strains having plasmids. Four strains appear to be have the same plasmid profiles as indicated in the banding patterns observed in fig. 1 (lanes 6, 11, 12 and 14) and the second clone appear between strains in lanes 4,7,10 and 13.

**III - Detection of virulence properties**

- The ability of *A. hydrophila* to cause a wide range of infections in animals and birds suggests a complex pathogenic mechanism that involves various factors (Lee *et al.,* 2000). Various putative virulence factors of *A. hydrophila* have been demonstrated in different types of samples collected at the same time to explain the process of pathogenicity.
- As shown in Table 2, 20 strains out of 26 examined isolates (76.9%) were positive for at least one of the putative virulence properties, 7/10 (70%), 9/9 (100%), and 4/7 (57.1%) from fish meal, clinically diseased and healthy birds respectively. There are 6 strains were negative to all

the virulence properties, 3 from the fish meal samples, 1 from clinically diseased birds and 2 from healthy ones.

**- Hemolytic assay.**

- Hemolysin is considered a putative virulence factor of *A. hydrophila* have been demonstrated in an effort to explain the process of pathogenicity. Out of 26 isolated strains 14 strains (53.8%) were β-hemolytic of which 6 strains (66.6%) out of 9 had been isolated from diarrheal cases followed by 50% from the strains (5 out 10) that isolated from fish meal samples. In contrast to 42.8% (3 out of 7) of the strains isolated from clinically healthy birds have this property.

**- Distribution of *aerA* and *ascV* genes.**

- PCR amplification of DNA from the examined *A.hydrophila* isolates using specific primers for aerolysin (*aerA*) and TTSS gene (*ascV*) resulted in the expected PCR products of 480bp and 710bp sizes respectively (Fig-1). The results showed 18 out of 26 examined strains (69.2.8%) harbouring (*aerA*), 9 strains out of 9 (100%) were positive from diarrheal cases and 3 out of 7 strains (42.8%) was positive from healthy/asymptomatic birds and 6 out of 10 strains (60%) examined from fish meal samples were positive for *aerA* gene.
- The gene encoding the putative virulence factor *ascV* gene was present more often in samples of clinically diseased birds in a 55.5% (5 out of 9) in contrast to isolates of clinically healthy birds 1 out of 7 (14.3%) was positive and 4 out of 10 (40%) strains of fish meal were positive (Table 2).

**- Plasmid of Virulence:**

- Plasmid of virulence is thought to be involved in the regulation of virulent genes that are coded for virulence with molecular weight 21kb (Majumdar *et al.,* 2009). Twelve strains out of 26 (46.15%) having plasmid of virulence (7 out of 9 strains (77.7%) have been isolated from diseased birds having 21 kb plasmids as shown in Fig.1 (lanes 1-4 and lanes 6-8), followed by 2 strains out of 7 (28.5%) that recovered from clinically healthy birds having plasmid of virulence (lanes 10 and 11) and 3 strains out of 10 (30%) of fish meal samples (lane 12 to 14) were having plasmid of virulence (Fig. 1).

**III - Relation between virulence traits presence and pathogenicity to chickens:**

- In a trial was made to determine the correlation between the presence of virulent traits (hemolysin production, *aerA* and *ascV* genes and

virulent plasmid) and pathogenicity to broiler chicks (1-day-old chicks) the following results were obtained:

- Mortality started at the 2nd day post-infection and cumulative mortalities of the infected groups were relatively high 80 % in group infected with the strain having the virulent traits in contrast to 50% mortality in group infected with the strain have not any of examined virulent traits. *A. hydrophila* was isolated from most organs examined (Table 5). Infected chickens showed depression, ruffled feathers as shown in fig.3. The lesions observed included congestion in the most of the internal organs as shown in Fig. 4 and unabsorbed yolk sac. No isolation or lesions were observed in control.

**III - Antibiotic resistance profiles of *Aeromonas hydrophila* isolates**

- Twenty six isolates of *Aeromonas hydrophila* (10 were isolated from fish meal samples, 9 isolated from diseased birds and 7 from clinically healthy birds) were tested against various types of antimicrobials using disk diffusion (Table 4).
- The antibiotic resistance patterns of 26 *A. hydrohila* isolates as the following; fish meal strains exhibited 90, 70, 60, 30, 20, 10 and 0% resistance to ampicillin, tetracycline, doxycyline, neomycin, ciprofloxacin, cefotaxime and gentamicin, respectively and 100, 87.5, 75, 31.3, 31.3, 18.8 and 25% of isolates were resistant to ampicillin, tetracycline, doxyccyline, neomycin, ciprofloxacin, cefotaxime and gentamicin, respectively from the clinical samples that isolated from birds. None of the isolates was found to be sensitive to erythromycin and ampicillin and amoxicillin either from strains isolated from fish meal and birds samples, in contrast to complete sensitivity was observed for gentamicin and colistin sulphate among fish meal samples.
- Eleven isolates of the examined *Aeromonas* showed multiple resistances to at least 3 up to 9 antibiotics with MAR index more than 0.2 as shown in Table 5.
- Regardless of the presence of plasmids, a high percentage of *A. hydrophila* strains were resistant to erythromycin, ampicillin and amoxicillin which indicates that resistance to these antibiotics is chromosomal.
- However, the presence of plasmids in other strains seemed to increase their antimicrobial resistance as indicated by presence of 8 multiple antibiotic drug resistance strains having plasmids in comparison to 3 plasmidless strains.

**Table 1:** The prevalence of *A. hydrophila* in different types of the examined samples

| origin of samples and Number | Samples positive for *Aeromonas* | Percentage of positive samples |
|---|---|---|
| Fish meal n =37 | 18 | 48.6 |
| Cloacal swabs from diseased birds n =50 | 9 | 18 |
| Cloacal swabs from healthybirds n =50 | 7 | 14 |
| Total | 34 | 24.8 |

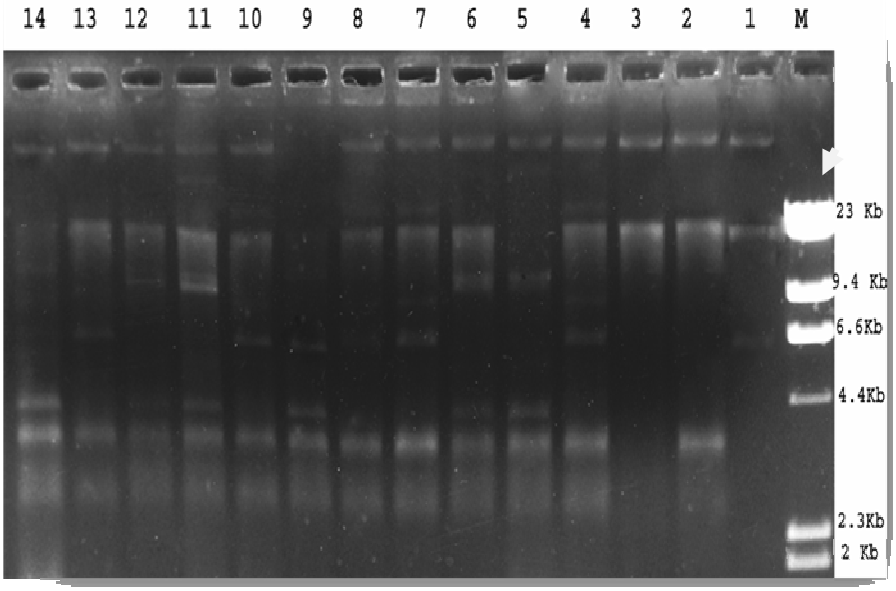


**Fig. 1:** Plasmid profiles of examined *A. hydrophila* separated by agarose gel electrophoresis Lane M λ *Hind* III digest molecular weight marker, Lanes 1-9 *A. hydrophila* isolated from diseased chickens, lanes 10-11 *A. hydrophila* isolated from clinically healthy birds and Lanes12-14 *A. hydrophila* isolated from fish meal. Arrow indicates on the plasmid of virulence.

**Table 2:** Distribution of virulence traits among *A. hydrophila* strains isolated from various types of samples

| Origin of sample | Number of samples screened | Virulence traits | | | |
|---|---|---|---|---|---|
| | | Hemolysin detection | Aerolysin (*aerA*) gene | *ascV* gene | Virulence plasmid |
| | | No. % of positive strains | No. % of positive strains by PCR | No. % of positive strains by PCR | No. % of positive strains |
| Fish meal | 10 | 5(50%) | 6(60%) | 4(40%) | 3(33.3%) |
| Cloacal swabs from diseased birds | 9 | 6(66.6%) | 9(100%) | 5(55.5%) | 7(77.7%) |
| Cloacal swabs from clinically healthy birds | 7 | 3(42.8%) | 3(42.8%) | 1(14.3%) | 2(28.6%) |
| total | 26 | 14(53.8%) | 18(69.2%) | 10(38.5%) | 12(46.1%) |

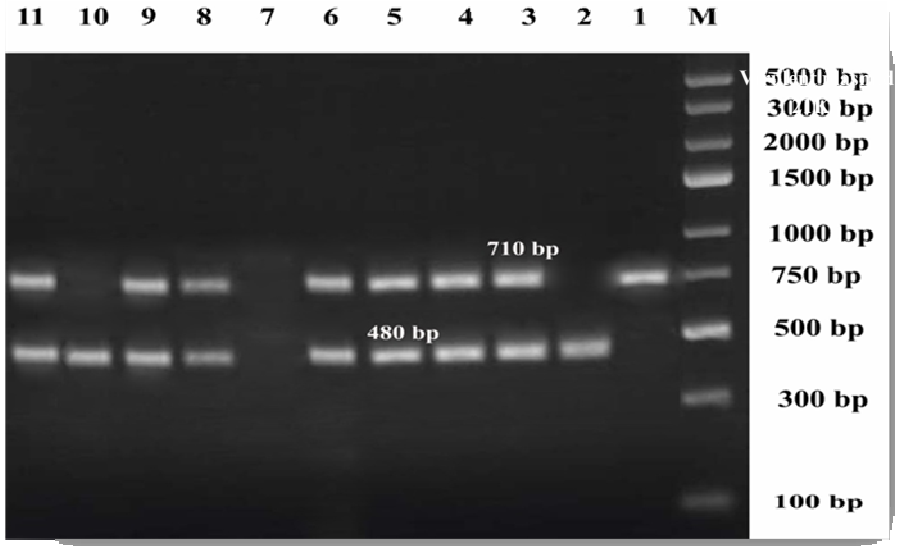


**Fig. 2**: Representative amplification products obtained using the multiplex PCR assay for detection of *A. hydrophila* in fish meal and broiler chicken samples. Lanes M, 100 bp DNA ladder; Lane 1, positive *A. hydrophila*, 710 bp of the *ascV* gene for TT3S gene and lane 2, positive *A. hydrophila* 480 bp of the *aerA* gene, Lanes 3 – 6, 8-9 and 11 positive for *aerA and ascV* genes.

**Table 3:** The mortality rate accompanied with *A. hydrophila* infected groups

| Group | Dose | No of infected chicks | Mortality | | | | | | Percentage of mortality |
|---|---|---|---|---|---|---|---|---|---|
| | | | Day[d]/Number of deaths | | | | | | |
| | | | 2 | 3 | 4 | 5 | 6 | 7 | |
| 1[a] | 1.5 X $10^9$ | 10 | 5 | 3 | 0 | 0 | 0 | 0 | 80 |
| 2[b] | 1.5 X $10^9$ | 10 | 3 | 1 | 1 | 0 | 0 | 0 | 50 |
| 3[c] | - | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a] Group infected with virulent *A. hydrophila* strain
[b] Group infected with avirulent *A. hydrophila* strain
[c] Control non infected group
[d] days after 1st day of infection

**Fig. 3:** Diseased infected chicks showing diffuse sleepy appearance and closed eyes

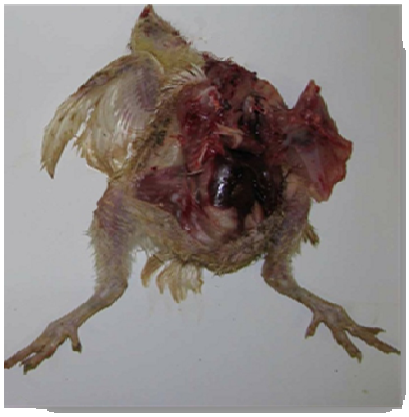

**Fig. 4:** Necroposied chicks showing congestion of internal organs

**Table 4**: *In vitro* susceptibilities of 26 selected *A. hydrophila* isolates from Fish meal broiler chickens samples.

| Antibiotic in (ug) | Zones of inhibition | | | | | |
|---|---|---|---|---|---|---|
| | Fish meal strains (n=10) | | | Clinical strains (n=16) | | |
| | R | S | %R | R | S | %R |
| Ampicillin (10) | 9 | 1 | 90 | 16 | 0 | 100 |
| Erythromycin (15) | 10 | 0 | 100 | 16 | 0 | 100 |
| Amoxycillin (25) | 10 | 0 | 100 | 16 | 0 | 100 |
| Ciprofloxacin (5) | 2 | 8 | 20 | 5 | 11 | 31.3 |
| Cefotaxime (10) | 1 | 9 | 10 | 3 | 13 | 18.8 |
| Doxycycline (30) | 6 | 4 | 60 | 12 | 4 | 75 |
| Tetracycline (30ug) | 7 | 3 | 70 | 14 | 2 | 87.5 |
| Gentamicin (10) | 0 | 10 | 0 | 4 | 12 | 25 |
| Neomycin (30ug) | 3 | 7 | 30 | 5 | 11 | 31.3 |
| Colistin sulphate (25 ug) | 0 | 10 | 100 | 0 | 16 | 100 |

The category 'S' means sensitive to antibiotic; 'R' means resistant. and each category was decided by zone diameter interpretive standards (CLSI, 2005).

**Table 5:** Multiple antibiotic resistance patterns of examined *A. hydrophila*

| Cutlrue No. | MAR index | Resistance Pattern | Plasmid acquisition |
|---|---|---|---|
| AH1 | 0.9 | A, Ax, E, C, CF, G, D, T, N | + |
| AH2 | 0.5 | A, Ax, E, C, CF | + |
| AH3 | 0.5 | A, Ax, E, C, CF | + |
| AH4 | 0.6 | A, Ax, E, C, G, D | + |
| AH5 | 0.6 | A, Ax, E, C, CF, D | + |
| AH6 | 0.7 | A, Ax, E, G, D, T, N | + |
| AH7 | 0.3 | A, Ax, E | - |
| AH8 | 0.4 | A, Ax, E, C | - |
| AH9 | 0.5 | A, Ax, E, C, G | + |
| AH10 | 0.5 | A, Ax, E, D, T | + |
| AH11 | 0.3 | A, Ax, E, | - |

A, ampicillin; Ax, amoxicillin; E, erythromycin; C, ciprofloxacin; CF, cefotaxime, G, gentamicin; D, doxycycline; T, tetracycline; N, neomycin; CO, colistin; MAR, multiple antibiotic resistance. The MAR index, is defined as a/b, where a represents the number of antibiotics to which the isolate was resistant, and b represents the number of antibiotics to which the isolate was exposed.

# DISCUSSION

*Aeromonas hydrophila* cause localized and systemic infections in avian species, as it caused septicaemia in turkeys (Gerlach and Bitzer, 1971), salpingitis in ducks (Bisgaard *et al*., 1995), diarrhea in water fowl (Efuntoye, 1995), conjunctivitis in pet parrots (Garcia *et al*., 1992), weight loss and diarrhea in cockatiels and canaries (Rosskopf and Woerpel, 1996) and diarrhea, feathers picking, sleeping, growth retardation and fluffing in different avian species (Jindal *et al*., 1993; Dorrestein, 1997; Ahmed 2004). *A. hydrophila* can cause localized or systemic infections in different avian species either alone or combination with other microorganisms (Barnes, 1997).

Our obtained overall isolation rate of *Aeromonas hydrophila* was 24.8% which is considered high than that observed with other studies that recorded by Glunder (1988); Kuplulu (2001); Ahmed (2004); Amal (2007); Moawad *et al.* (2009); Mahmoud and Tanios (2008) who recovered *A. hydrophila* in incidences of 3.6%, 82.9%, 2.33 %, 15%, 3.2% and 6.8% respectively.

In this concept, the isolation rate from fish meal showed 18 out of 37 samples were positive to *A. hydrophila* (48.6%). This higher isolation rate may be suggested due to inadequate sanitizing procedures of fish meal manufacturing that may contribute to dissemination of *Aeromonas* to broiler chickens when be added to ration components (Kirov, 2001).

This variation in the prevalence of *Aeromonas hydrophila* among similar studies conducted on chicken may be attributed to; i- the variation in fecal samples number ii- different methods used for isolation of *Aeromonas* iii- ration composition iv- level of sanitation vi- time of collection of samples in season of winter give a good environment for viability of *Aeromnas hydrophila* that may extend to 25 day (Awaad *et al.,* 2011) vii- Different antimicrobial agents being routinely used in farms.

Also to determine the potential risks of fish meal associated with the exposure of chickens to *A. hydrophila*, epidemiological tracking is required. Conventional methods used for bacterial identification are tiresome, time consuming and not fit for mass scale screening of bacteria. Microbial molecular genetics is gaining popularity in recent times as an essential tool in the classification of the microbes. The potential of plasmid analysis for characterization and evaluation of heterogeneity in *A. hydrophila* was assessed.

Our study revealed that fourteen out of 26 examined isolates (53.8%) carried detectable plasmid DNA as shown in Fig 1. Plasmid profile analyses revealed that all plasmid-containing strains have multiple plasmids from 3 to 6 in number that ranging in size from 2.7-56 Kb (Fig. 1). There are two common clones between strains having plasmids. Four strains appear to be had the same plasmid profiles as indicated in the banding patterns observed in Fig. 1 (lanes 6, 11, 12 and 14) and the second clone appear between strains in lanes 4,7,10 and 13.

The frequency of occurrence and the detection of plasmids among the *Aeromonas* were in broad agreement with those of other workers who found plasmid prevalence in *Aeromonas* have been 15 to 94%, with most of the isolates harbouring small sized plasmids (Chaudhury *et al*., 1996; Brown *et al*., 1997).

The exact role of *A. hydrophila* in disease still remains in a state of flux, it is accepted as an opportunistic pathogen for chickens. The pathogenesis of Aeromonas infection is considered complex and involves a number of virulence factors. Several factors of *Aeromonas* species, including cytotonic and cytotoxic enterotoxins, protease, hemolysin, and siderophores, have been considered to play a role in for intestinal infections in human beings. Recently, type III secretion system (TTSS), common in pathogenic strains of gram-negative bacteria and were identified in *Aeromonas* species (Chaco´n *et al.,* 2004).

No studies have investigated the potential virulence factors among strains causing infections in broiler chickens. It is known that pathogenic isolates of *A. hydrophila* secrete aerolysin toxin that causes the lysis of the RBCs. In the present study, as shown in Table 2, 14 isolates out of the 26 were phenotypically positive for hemolysis on the blood agar medium by hemolytic activity detection, as a variable halo diameter between 0.5 and 2 mm. However, 18 isolates were positive for presence of 480 bp aerolysin gene by PCR amplification. It is possible that some of the isolates though positive for aerolysin gene did not cause hemolysis on blood agar either due to disruption of gene or due to mutation in gene (Wang *et al.,* 2003).

The higher concentration of haemolysins of *A. hydrophila* in our strains isolated from clinically diseased birds agrees with the results obtained by other author (Abulhamd, 2009) on clincial and environmental strains. The haemolytic activity is strongly associated with enterotoxin production in members of the *Aeromonus* genus (Burke *et al.,* 1983).

The presence of aerolysin toxin gene was demonstrated in all the isolates recovered from diseased birds, whereas 42.8% of *A. hydrophila* isolates recovered from apparently normal birds showed the presence of aerolysin toxin gene. Therefore it can be safely assumed that aerolysin plays a role in the bacterial pathogenicity along with other virulence factors as reported by Chu and Lu, 2005 who considered the aerolysin is one of the major virulence factors in gastroenteritis.

The T3SS is considered a virulent trait that correlates with bacterial pathogenicity, and its presence can be used as a general indicator of virulence (Stuber *et al.,* 2003). Globally, 38.5% of *A. hydrophila* isolates presented this gene. The low incidence of this gene in strains isolated from clinically healthy birds is remarkable and different from the incidence in the diarreheic samples (Table 2). This agrees with the low virulence reported for this gene, in contrast, to all extraintestinal isolates of *Aeromonas* had this gene (Wang *et al.,* 2003).

In fact, the discovery of T3SS and toxin genes in such a high proportion of clinical strains could raise these microorganisms to the category of primary pathogens. So we need a further characterization, by sequencing, of the TTSS in *Aeromonas* is ongoing and will enable its full comparison with the TTSS of other microbes. This is key information for a better understanding of the pathogenicity potential and virulence mechanisms of *Aeromonas*. In agreement with previous studies (Krzymińska *et al.,* 2001; Sechi *et al.,* 2002), we found a heterogeneity in the distribution of examined virulent genes among the tested strains

Other virulent factor was analyzed; plasmid of virluence was detected among 12 out of 26 examined strains (46.1%) with highest percentage of possession was noticed among 7 out of 9 strains (77.7%) were recovered from diseased diarrheic birds followed by 3 strains out of 10 (30%) of fish meal samples (lane 12 to 14) were having plasmid of virulence then 2 strains out of 7 (28.5%) were ioslated from clinically healthy birds (lanes 10 and 11). These mentioned frequencies of virulence plasmid occurrence among A. hydrophila were in broad agreement with other workers as has been observed by Borrego *et al.* (1991).

In our experiment that done to explain the role of possession of virulent traits toward the pathogenicity of *A. hydrophila* for 1-days-old chicks, we observed the isolate bearing virulent traits could infect baby chicks more efficiently and multiplied in internal organs causing clinically evident infections with 80% mortality more than that observed

by chicks infected by non virulent strain that caused 50% mortality. In accordance with this result, Wong *et al.,* 1998 mentioned that all *A. hydrophila* isolates with haemolysin positive genotype were virulent in the suckling mouse assay model. Studies have demonstrated that aerolysin mutant strains were less virulent in assays of toxicity in vivo and in vitro (Fadl *et al.,* 2007). A previous report Hanes and Chandler 1993 has shown that the presence of plasmids in clinically important bacteria increases their virulence.

At necropsy; the experimentally infected chicks revealed congestion of liver and heart as shown in fig. 4. Further reports run in parallel with our results, Gerlach and Bitzer (1971) described septicaemic condition in commercial turkeys aged 3- 16 weeks that was attributed to *A. hydrophila* infection with 10-30% morbidity rate and 1-5% mortality rate. Furthermore, Shane and Gifford (1985) reported that 2-4 day-old experimentally infected chicks were highly susceptible to *A. hydrophila* exposure via subcutaneous, yolk sac or intracerebral routes with mortality rate ranging from 80-100%. El-Khashab (2001) experimentally infected 2 and 5 day–old chicks with *A. hydrophila* organism via subcutaneous or oral inoculations. The results revealed that some chicks died acutely while chicks that died later demonstrated a transitory period of depression characterized by ruffled feathers and pasty vent before death with mortality rate ranging from 60-100%. She also observed generalized congestion of liver, spleen, lungs, kidneys, intestine (especially duodenum) with severe haemorrhagic enteritis. Ahmed (2004) found that *A. hydrophila* induced acute death within 24 hrs of the inoculated chicks with 100% mortality rate after yolk sac inoculation and 86.6% after subcutaneous inoculation. The most predominant lesions findings were generalized venous congestion, peticheal haemorrhages on the liver, omphalitis, enteritis and nephrosis. No macroscopic lesion observed, except congestion of internal organs, was seen (Akkoc *et al.,* 2008).

The extensive use of antibiotics in broiler farms, either to prevent or cure broiler chicken diseases, both as feed additives or dissolved directly into the water, has resulted in increasing antibiotic resistance among pathogenic bacteria, so our isolates tested for antibiotic susceptibility.

In comparing the antibiotic profile as shown in Table 4, the *A. hydrophila* isolated from fish meal encountered in this study were all susceptible to gentamicin in contrast to 78% from clinical isolates. They

all show a high sensitivity to ciprofloxacin, cefotaxime, gentamicin and neomycin. All the isolated strains were resistant to amoxicillin, erythromycin and colistin sulphate and high resistance level against tetracycline and doxycycline esp in clinical strains than that observed with fish meal samples. Oxytetracycline resistance frequencies are high among *Aeromonas* isolate as shown with Schmidt *et al*. (2000); Goni-Urriza *et al*. (2002) found as many as 49% tetracycline-resistant *Aeromonas* species isolated from European rivers. The result conforms to this study which shows that *Aeromonas* species are poorly susceptible to tetracycline.

In a study by Ko *et al*. (1996), most of the isolates were resistant to first-generation quinolones (pipemidic acid and oxolinic acid) but clinically susceptible to fluoroquinolones from pefloxacin (54%) to ciprofloxacin (98%). This study confirms the susceptibility of *Aeromonas* species to ciprofloxacin, pefloxacin and ofloxacin. *Aeromonas* species isolated in this study were all 100% susceptible to these antibiotics. Antibiotic resistance frequencies and profile varied according to the source of the strains

Subashkumar *et al.* (2006) reported that *A. hydrophila* exhibited resistance to erythromycin and tetracycline (71.4% each) and exhibited susceptibility to gentamicin (76.2%). All the isolates were resistant to ampicillin as has been reported earlier (Schmidt *et al.,* 2001). Amal (2007) also reported that gentamicin was the most effective drug (100%) while neomycin was moderately sensitive (80%). Decreased susceptibility to third- generation cephalosporins (Fosse *et al.,* 2003) and susceptibility to aminoglycosides, fluoroquinolones and carbapenems (Vila *et al.,* 2003; Sader and Jones, 2005) were reported previously and are in accordance with our results.

In this study, *A. hydrophila* showed a resistance pattern to different antibiotics that was previously reported by several authors (Akinbowale *et al.,* 2007; Cattoir *et al.,* 2008; Ribeiro *et al.,* 2010).

As shown in Table 4, we noticed apparently that most common antibiotic, indiscriminately used by farmers, have given rise to resistant bacterial strains as mentioned by Vila *et al.* (2003). Therefore, controlled, in-depth studies are needed to determine the effect of antimicrobial therapy on the microbial ecology in broiler chicken farms in Assiut province.

**In conclusion, findings of present work were summarized as:**

- Infection with *A. hydrophila* is widely disseminated in fish meal and clinical samples (diarrheic and apparently healthy broiler chickens) and was significantly more common in strains isolated from diarrheic cases.

- Based on Plasmid profiling, there is a two shared clones of *A.hydrophia* among fish meal and clinical samples isolates that suggest there is fish meal-to-chicken transmission, and that exposure to *Aeromonas* can lead to diseases in broilers.

- *A. hydrophila* strains recovered from diarrheic birds contained higher virulence traits percentages than that observed from strains isolated from fish meal and clinically healthy birds. Thus, it seems possible that the virulent *A. hydrophila* have much greater potential to cause disease in broiler chickens and may raise this microorganism to the category of primary chicken pathogens.

- There is a good correlation between the presence of a virulent traits and pathogenicity of *A. hydrophila* to one-day-old chicks. Screening o f specific T3SS and aeolysin genes appear to be the most effective way of detecting and characterizing *Aeromonas* virulence factors.

- Bacteria isolated from the intestine of broilers seemed to be more resistant than bacteria from fish meal that led us to the conclusion that these *Aeromonas* isolates may serve as a reservoir of antibiotic resistance genes, and the resistance may be transferred to other bacteria in the environment of farms and continuous monitoring and evaluation of emerging antibiotic resistance in *A.hydrophila* is of great importance in enforcing adequate control measures including policy imperatives on antibiotic usage. Gentamicin, cefotaxime and ciprofloxacin are suitable antibiotics that can be used in the treatment of *A. hydrophia* associated infections.

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