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Distribution, involvement and plasmid characterization of *Aeromonas* spp. isolated from foodstuffs and human infections

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ABSTRACT

A survey was carried out in Ismailia governorate Egypt to ascertain the distribution of *Aeromonas* spp in different food sources and human infections. Thirty five foodstuffs; chips, baby food, cake, tea creamer, canned drinks, biscuits, pre/semi-cooked meals and raw vegetables, were investigated. Out of 138 strains isolated from all the studied food sources and picked up from different prepared culture media, only 81 isolates comprising 59% were identified as *Aeromonas* spp. Three species were confirmed *A. hydrophila* (45 isolates), *A. punctata* (22 isolates) and *A. salmonicida* (14 isolates). Ninety eight human hospital cases suffering from five different types of infections were investigated. The studied cases included fourteen otitis, seven conjunctivitis, seven vaginitis, fourteen renal failures, seven pyelonephritis and forty-nine anemic people (7 dL/g < haemoglobin < 12 dL/g). As many as 74% of the 273 isolates from these cases were identified as *Aeromonas*, in a distribution pattern of; *A. hydrophila* (45%), *A. punctata* (34%) and *A. salmonicida* (21%). All isolated strains found, more or less, involved in all infections, except conjunctivitis. *A. hydrophila* dominated in renal failure (83%) and pyelonephritis (67%) cases, while *A. salmonicida* dominated in vaginitis ones. Antibiotic resistance profiles of representative selected isolates (9 isolates) to 5 antibiotics namely; ampicillin, chloramphenicol, tetracycline, streptomycin and vancomycin, were plotted. Fortunately all the isolated strains showed sensitivity to the tested antibiotics. Two ampicillin/vancomycin resistant *A. hydrophila* isolates of clinical origin were examined for the presence of plasmid DNA. Each of them found to contain only one plasmid with molecular size of 32 and 24 kbp, respectively. Transformation coefficient, resistance expression within *E. coli* HB 101 and *E. coli* DH 1 and curing of the plasmids, indicated that the ampicillin- resistance of these two isolates is plasmid-linked. The study concluded a wide distribution of this, supposed to be opportunistic, as a probable infectious organism, amongst many Foodstuffs, as well as being involved in variable human infections in Ismailia, Egypt.

Keywords: *Aeromonas* sp, Distribution, Plasmids, FoodStuffs, Opportunistic Human Infections.

INTRODUCTION

Although the organisms are considered natural inhabitants in the aquatic environment, *Aeromonas* spp. can be isolated from a variety of foods, including meat, poultry, milk and milk products, fish and shellfish, and vegetables (Knochel & Jeppesen, 1990 and Nishikawa & Kishi 1998). There is an increasing amount of evidence that at least some strains of *Aeromonas* spp. are involved in the etiology of gastrointestinal diseases in human beings and that they cause serious infections in immunocompromised patiants (Villari *et al*. 2000). In recent studies *Aeromonas* have been associated with three types of human illnesses including extra-intestinal, wounded, and gastrointestinal infections. The spectrum of symptoms includes gastroenteritis, cellulitis, meningitis, backeraemia soft-tissue infection, peritonitis and bronchopulmonary infections (Pin *et al.* 1997 and Tanios *et al.* 1997). *Aeromonas* species, specially motile ones, have long been recognized as

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primary pathogens, (Khashe *et al.* 1996) and increasingly known as aetiological agent of disease syndromes such as gastroenteritis, (Altwegg *et al.* 1991) otitis, (Vizmanos *et al.* 1994) vaginitis and kidney infections including pyelonephritis, (Khedr *et al.* 1995 and Rusin *et al.* 1997 a&b). *Aeromonas* species are also noted to be important pathogens of pediatrics and immuno-compromised patients causing diarrhoea or dysentery leading to dryness and even more to death in many cases, (Essers *et al.* 2000). This initiated the present study so as to investigate the distribution and degree of involvement of *Aeromonas* species in different food sources and human clinical infections. Special reference was paid to *A. hydrophila, A. punctata and A. salmonicida*. Also to investigate any relation between the antibiotic-resistance of *Aeromonas* species isolated and the presence of plasmids. The contribution degree of these plasmids, if there is any, in antibiotic-resistance and the characters of these plasmids including transferability and expression capacity was also targeted.

MATERIALS AND METHODS

Microbiological Studies

Food Sampling and Sample Processing

Thirty five food sources were chosen and classified as follows: chips, baby food, cake, tea creamer, drinks, biscuits, cooked food, semi cooked foods, raw food and vegetables. Obviously, these foods are of particular concern, because they are greatly demanded by all people including children. Samples (10 gm) were weighted aseptically in stomacher bags containing 90 mL of alkaline peptone water (APW, 5% peptone, pH 8.6), and homogenized in a blender for 2 min. After decimal dilutions, 0.1 mLfrom each dilution was spread onto starch ampicillin agar media. Lugol iodine (I2 5 g, potassium iodide 10 g in 100 ml distilled water) applied to 24 h incubated starch ampicillin agar plates to confirm Aeromonas colonies with clear zones around them. Plates were incubated for 24 h at 30 ºC (Villari *et al.* 2000).

Human Clinical Infection Sampling and Sample Processing

Ninety eight clinical volunteers suffering from different types of infections were investigated. Otitis, conjunctivitis and vaginitis cases were sampled as routinely using sterilized cotton swabs. Swabs were, separately, washed in 100 ml alkaline peptone water (APW, 5% peptone, pH 8.6), then 1 ml aliquots from the resulting bacterial suspensions were dispensed onto nutrient, MacConkey and starch ampicillin agar plates following spread plate method. Lugol was applied to 24 h incubated starch ampicillin agar plates to confirm Aeromonas colonies with clear zones around them. Urine samples were collected as described by Collins *et al.* (1998) from the 21 renal cases included 14 renal failure and 7 pyelonephritis cases. The collected mid-stream urine samples in 100 sterile glass vials were plated, 200 µl, on the same previously described culture media. Stool samples (1 g) from 49 anemic cases (7 dL/g < haemoglobin < 12 dL/ g) were extracted each in 100 ml saline solution 5 % NaCl and 200 µl of 10-3 serially diluted extracts were dispensed onto nutrient agar and other culture media used in the study. All plates were incubated face down and the bacterial were allowed to grow at 35 °C for 24-48 h prior to enumeration and further identification.

Identification of Bacteria

Identification of well-isolated pure colonies proceeded to the specific level adopting Holt *et al.* (1994) based on biochemical tests. This involved the glucose oxidation/ fermentation, lactose fermentation, oxidase test, nitrate reduction, indole reaction, urease production, Voges Proskaure, arginine and ornithine utilization as carbon sources.

Antibiotic Resistance Studies

All experiments were conducted using the original stock cultures nutrient agar to avoid the spontaneous loss of antibiotic resistance sometimes associated with frequent subculturing (Barry 1991 and Mckeon *et al.* 1995). Antibiotic resistance was assayed using a modified Kirby-Bauer disk diffusion method (Robert *et al.* 2003). Bacterial strains were inoculated into 5 mL of sterile broth and incubated at 37oC for 18 hours. A loopful was then diluted in 5 mL sterile phosphate buffered saline (PBS) and seeded onto Muller-Hinton medium. After the inoculum has dried antibiotic discs were applied to the inoculated medium with sterile forceps and pressed down gently to ensure even contact. Plates were incubated at 25oC for 48 hours and antibiotic resistance represented by the presence or absence of clear zones around the growth of bacteria (Collee *et al.* 1996 and Collins *et al.* 1998). Antibiotic Standard discs "Oxoid" concentrations were shown in table (1). These antibiotics were chosen to fulfill and used in the prevention and/or treatment of human ocular infection (Mckeon *et al.* 1995). Diameter in mm of clear zones surrounding the antibiotic discs indicating bacterial growth inhibition was measured after 24 hours incubation at 37oC. Bacterial isolates were characterized as "resistant', "intermediate" or 'sensitive" according to the manufacture guide lines (Oxoid Manual 1980). Minimum inhibitory concentrations (MICs) were determined by the broth dilution method: A stock solution was used to prepare a range of antibiotic concentrations in 1mL of Muller-Hinton broth. Bacterial suspension (1 mL containing approximately 105:5×105 cells) of each resistant isolate to a particular antibiotic was used to inoculate each concentration and growth of bacteria was checked after overnight incubation at 37oC (Lorian 1986).

Plasmid Studies

Plasmid Isolation: LB broth plus appropriate antibiotics in culture tubes with each individual bacterial colony were shaked at 37oC overnight. Cell pellets are resuspended in 100 µL alkaline extraction with solution I composed of 50 mM glucose, 25 mM tris (pH 8.0) and 10 mM EDTA, combined with fresh 20% sodium dodcyle sulphate (SDS), solution II by combining 1 mL 2 M NaOH, 0.5 mL 20% SDS and 8.5 mL H_2O. 150 µL of ice-cold solution III (3 M KOAC were added, brought to pH 5.5 by adding glacial acetic acid) to each tube. A white precipitate of denatured proteins and cell debris was formed. Two handerds microliter phase separation mixture of phenol-chloroform (1:1) was added to each Tube. Closed microfuge with inverted tubes was centrifuged several times to mix the phases well. The samples were centrifuged for1 minute in a microfuge. Using a pipettor or a Pasteur pipet, the upper (aqueous) phase was transferred to new microfuge tubes. The DNA was precipitated using 300µL cold isopropanol to each tube. Fresh TE [50 mM Tris (pH 8.0); 20 mM Nacl and 5 mM EDTA] plus digestion of RNA using RNase by adding 20 µL (5 mg/mL) RNase in TE buffer was prepared. A 5µL of 3 M sodium acetate and 125µL cold ethanol were added to DNA. Samples were stored at –20 oC indefinitely. The pellets were resuspended in 40 µL 1 mM Tris and 0.1 mM EDTA, pH 7.5 (Sambrook *et al.* 1989).

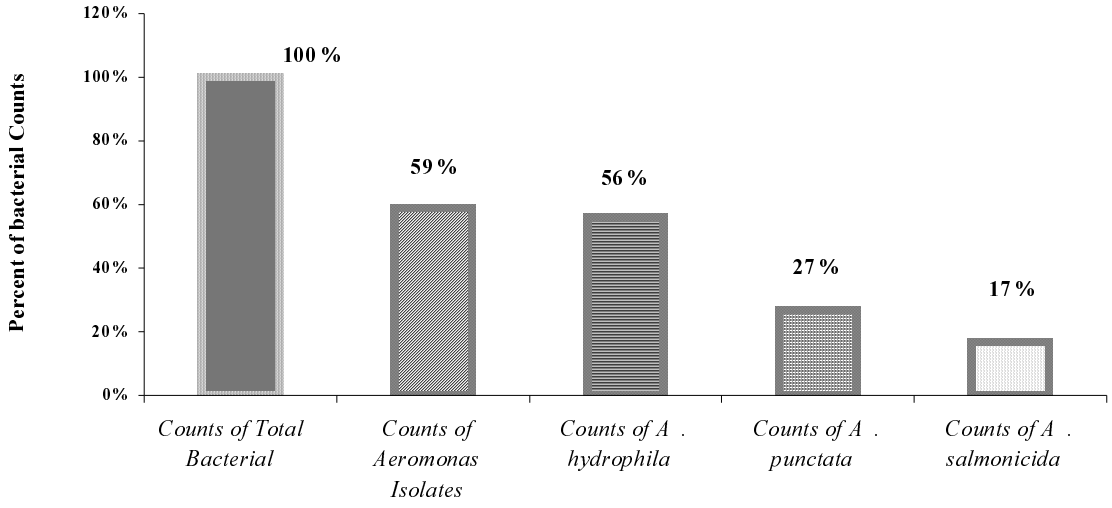
Plasmid DNA Concentrations and Purity Determination: Concentrations and purity of DNA were estimated spectrophotometrically using Spectro 22, Labo Med, Inc., and USA. The concentration of plasmid DNA was determined at 260 nm. The purity of plasmid DNA was calculated by the ratio of absorbance at 260 nm and 280 nm (Sambrook *et al.* 1989).

Agarose Gel Electrophoresis of the Plasmid DNA: Agarose gel electrophoresis was carried out using the tris-borate EDTA buffer (TBE). Gels were prepared by adding 1% agarose and 5 µL ethidium bromide (stock solution of 10 mg/mL) to the TBE buffer as described by Hammad & Dora 1993.

Curing and Transformation Protocols: Serial dilutions of acridine orange were used for curing (Ramteke *et al.* 1990). Two different ampicillin-senstive and plasmid-free E.coli strains; DH1 and HB 101 were used as recipient cells in the transformation experiments using Chlorides mixture method exactly as described by Sambrook *et al.* (1989).

RESULTS

Microbiological Studies: Total viable bacterial TVB counts cfu/100 mL was very high in almost all kinds of investigated food sources. For all the food sources out of the 138 isolates picked up from different prepared culture media. Only 81 isolates comprising 59% were identified as *Aeromonas* spp. Three *Aeromonas* species were confirmed *hydrophila* (45 isolates), *punctata* (22 isolates) and *salmonicida* (14 isolates). The participation percentage of each of these three species in the studied food sources was as shown in Figure 1.



Out of the 273 isolates picked up from different prepared culture media in this study, only 203 isolates comprising 74% were identified as *Aeromonas* spp. Three species were confirmed; *A. hydrophila* (105 isolates), *A. punctata* (77 isolates) and *A. salmonicida* (21 isolates). The participation percentage of each of these three species in the studied human infections was as shown in Figure 2. The distribution pattern percent of these species in each infection site was as plotted in Figure 3. *A. hydrophila* showed the highest participation percentage (46%) , distributed in renal failure (83%), pyelonephritis (67%) and in anemia (14%) (Figure 3). Forty-two *A. punctata* representing 86 % of its total isolates were involved in anemia cases. Vaginal infections were dominated by *A. salmonicida*. *A. salmonicida* comprised only 33% of isolates from otitis cases and 17% from pyelonephritis cases. Pyelonephritis infection was the only that harbored all of the three investigated *Aeromonas* species in the present study, although *A. hydrophila* was dominated (Figure 3). Isolation experiments revealed that the best growth of *Aeromonas* from stool specimens was on nutrient agar medium. Recovery of *Aeromonas* from ear (otitis cases) and vagina (vaginitis cases) better isolated on starch ampicilin agar. MacConkey and starch ampicilin agar media are preferable when isolating *Aeromonas* from urine samples of pyelonephritic as well as renal failure patients. Although the bacterial counts from the studied eye infections (7 conjunctivitis cases) were

comparatively high representing (26% of the total studied isolates), non of them was identified as *Aeromonas*. The majorities were found gram-positive cocci.

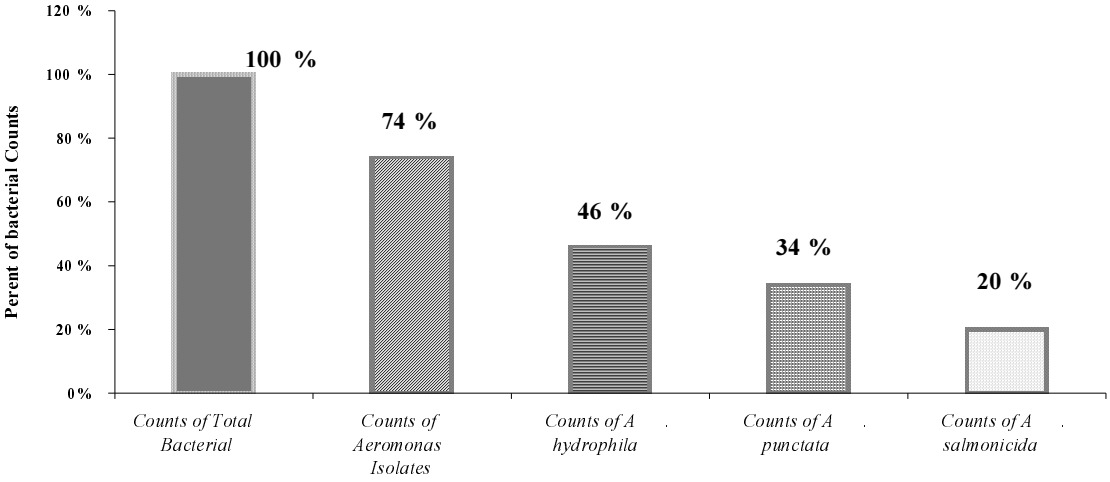


Fig. 2: Participation of the studied *Aeromonas* spp. percent in human clinical infections.

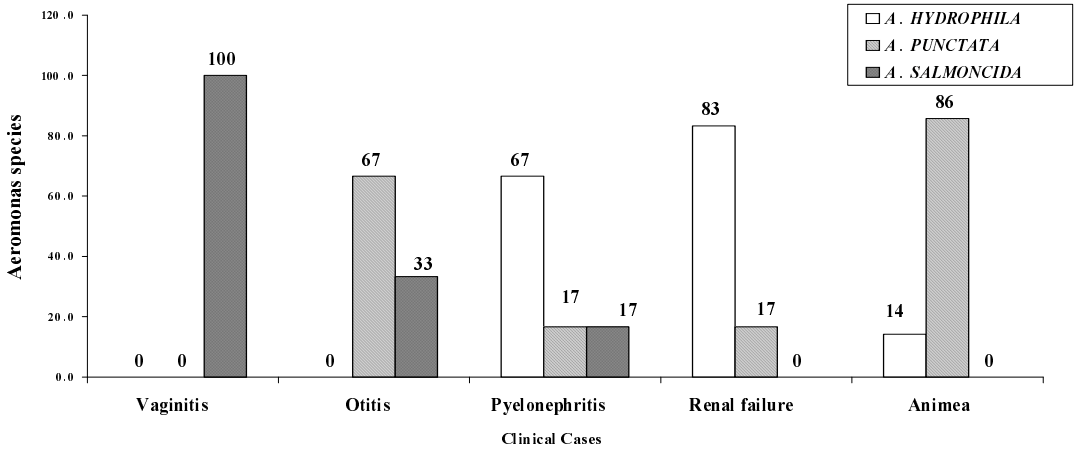


Fig. 3: The contribution of each of the three investigated *Aeromonas* species; *hydrophila, punctata* and *salmonicida* in the 98 studied clinical cases.

Antibiotic Resistance Studies: Representatives of the identified species (9 isolates) were chosen to perform sensitivity test against five commonly used antibiotics belongs to different families: ampicillin, choramphenicol, tetracycline, streptomycin and vancomycin. The results of the disk diffusion susceptibility testing (Table 1) showed that all of the isolates were sensitive to antibiotics, but only two human clinical isolates *A. Hydrophila* 25 and 26 were resistant to ampicillin and vancomycin. From the results we has taken two resistant isolates to make minimum inhibitory concentrations (MICs) and the plasmid. MICs of the tested antibiotics against the two isolates *A. Hydrophila* 25 and 26 were 16 µg/mL and 32 µg/mL respectively.

Plasmid Studies: Plasmid profiles of the two antibiotics-resistance bacterial isolates under study were determined. The two bacterial isolates were found to contain one plasmids (Table 2 and Figure 4). Molecular sizes of the detected plasmids were found 32 kbp in *A.hydrophila* 25 and 24 kbp in *A. hydrophila* 26 (Fig. 4). Concentration and degree of purity of the plasmid $DNA_{(S)}$ were as in table 2. Plasmid curing in combination with MIC determination revealed that of the isolated plasmids resistance to vancomycin is plasmid-

linked while for resistance to ampicillin were found plasmid and chromosomal linkage. *E. coli* HB 101 that is plasmid-free and ampicillin sensitive strain, transformed with each of the two isolated plasmids, in separate trails while *E. coli* DH 1 strain was not. Strain HB 101, received the plasmid, and expressed the ampicillin resistance to $MIC_{(S)}$ very close to that of the original donor (Table 2).

Table 1: Antibiotic resistance profile of the bacterial isolates from ocular infection.

Aeromonas Isolates	A=Ampicillin 10 µg	C=Chloramphenicol 30 µg	TE=Tetracycline 10 µg	S=Streptomycin 10 µg	VA=Vancomycin 30 µg
A. salmonicida 3	+	+	+	+	+
A. Punctata 8	+	+	+	+	+
A. Hydrophila 9	+	+	+	+	+
A. salmonicida 13	+	+	+	+	+
A. Hydrophila 29	+	+	+	+	+
A. Punctata33	+	+	+	+	+
A. Hydrophila 66	+	+	+	+	+
A. Hydrophila 25	-	+	+	+	-
A. Hydrophila 26	-	+	+	+	-

Abbreviations:. **-** = Resistant, + = Sensitive

Table 2: Characterization of isolated plasmids including size, concentration, purity and MICS of the original and transformed isolates.

Aeromonas Isolates	Plasmid Size (Kbp)	Conc.(µg/mL) /Purity	MIC Mother Cell	(µg/mL) Transformed *E.coli*
A. Hydrophila 25	**32**	**320/1.3**	**16**	**16**
A. Hydrophila 26	**24**	**320/1.8**	**32**	**32**

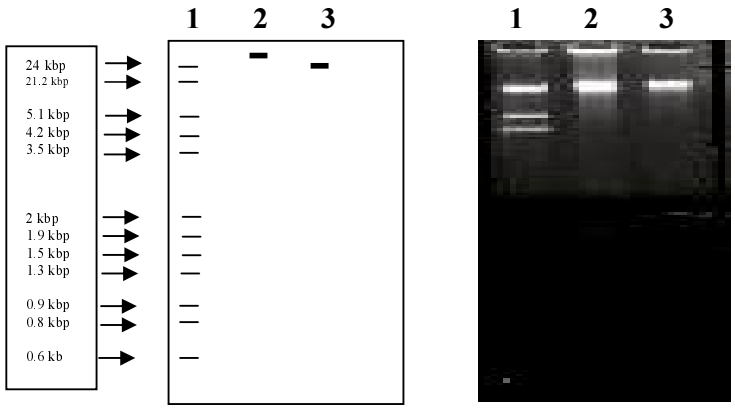


Figure 4: Molecular size of recovered plasmids against Lambda (λ) DNA digested with Hind III + EcoR I; as a reference, lane 1, pure plasmids preparation from *A. Hydrophila* 25, lane 2, from *A. Hydrophila* 26, lane 3.

DISCUSSION

There are relatively few published cases in which *Aeromonas* spp. has been associated with foodborne gastroenteritis. Suspect foods presumably were either inadequately cooked before consumption or consumed directly or after minimal cooking (Villari *et al*. 2000). The contamination by *Aeromonas* spp. of baby food and ready-to-eat foods is of particular concern, because these products receive no further cooking and therefore may represent a major source of infection for human being. The results of this study indicate that

Aeromonas spp. are common in food sources consumed in Egypt confirming the finding of other surveys conducted in other countries (Knochel & Jeppesen 1990 and Villari *et al*. 2000). *Aeromonas* spp in this study, comprised 74% of the total bacterial counts isolated from different human clinical infections. This presented genus *Aeromonas* as the most common opportunistic human pathogen that is not limited to certain sites or acompanioning specific disease agents. Altwegg *et al.* (1991), Vizmanos *et al.* (1994) & Rusin *et al.* (1997a&b) are some of the researchers who got similar results and alarmed the increasing involvement of *Aeromonas* in human infections. More precisely, *A hydrophila*, *A. punctata* and *A. salmonicida* occupied respectively, in the present work, the first three positions amongst *Aeromonas* species. Although *A. hydrophila* had been extensively studied at different levels in human health risks, (Diab 1989, Carnahan & Joseph 1993, Khedr *et al.* 1995, Hanninen & Siltonen 1995, Kersters *et al.* 1996, Khashe *et al.* 1996, Massa *et al.* 1999, Xiang *et al.* 1999, Essers *et al.* 2000 and Kozinska & Antychowicz 2000) it showed here its superiority (83 %) in pyelonepheritis and other renal problems including renal failure. Culture preservation in refrigerators (chilling), comprises a stress factor that affect the biochemical activities of these cultures. Response of different *Aeromonas* isolates to chilling considered as one of the important on which analysis was based and found differential, Kersters *et al.* (1995), Littla *et al.* (1997) and Uddin *et al.* (1997) recognized this, but they neither included it in their data matrix nor relay on it in their analysis. The study gave also a shortcut for the culture media choice suitable for the isolation of different *Aeromonas* spp. based on the site and type of infection. The occurrence of multiple antibiotic resistant (MAR) organisms has attracted the attention of many workers to the phenomenon of transferable drug resistance factors between bacteria (Pathak & Gopal, 1994). Increasing antibiotic resistance is a factual problem. It occurs from the infected patient who respond poorly to treatment and in a hospital, which may perform infection control and prevention programs. Clinical presentations are not diagnostic of the cause. Microbiological analysis with cytology, cultures and microbial sensitivities is mandatory. In recent studies plasimd-linked resistance, especially for pathogenic bacterial isolates, are still of critical importance (Bekowitz 1995 and Demain & Davies 1999). Transformed *E. coli* HB 101, initially expressed ampicilllin resistance at almost the same MIC of the original mother strain; the strain that originally bear the plasmid, but gradually lose resistance resulting in ampicillin sensitive and plasmid free *E. coli.* Plasmid instability has been reported to be due to several reasons such as fragmentation, mutation or drop in copy number (Russell 1998). Detailed characterization of these plasmids, genome and level of molecular heterogeneity of *Aeromonas* spp. is needed in future for more understanding about transferability, gene expression and stability.

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الملخص العربي

إنتشار ودرجةِ تدخل وخصائص البلازميد لأنواع أيروموناس المعزولة من مواد غذائية وحالات مرضية بشريه

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تم عمل مسح في محافظة الإسماعيلية–مصر بهدف معرفة توزيع ودرجةِ تدخل جنس بكتيريا ايروموناس المعزولة من مصادر الطّعام المختلفةِ ومن بعض الحالات المرضية المتنوعة في الإنسان. تم تجميع خمسة وثلاثون عينة طعام شملت بطاطس، طعام رضّع، كعك، شاي , أشربة محفوظة، بسكويت، وجبات قبل / نصف مَطْبُوخة وخضار. من ١٣٨ عَزلَه من كل مصادر الطّعام المدروسةِ والتى تم تجمعيها من مواد غذائية مختلفةِ. تم تعريف ٥٩ % منها على أنها ايروموناس. توزعت بين أنواع ثلاثة هى؛ هيدروفيلا ٥٦ %، بنكتاتا ٢٧ % و سالمونيسيدا ١٧ %. و أيضا تمت دراسة عينات من ٩٨ حالة (افراد متطوعة) تعانى من أمراض مختلفة بغرض الكشف عن دور و توزيع وانتشار أنواع جنس بكتيريا ايروموناس فيها. الحالات تحت الدراسة شملت ١٤ حالة التهاب الاذن، ٧ حالات التهاب ملتحمة العين، ٧ حالات التهاب المهبل، ١٤ حالة تعانى من الفشل الكلوى، ٧ حالات التهاب كلوى و ٤٩ حالة أنيميا (تتراوح فيها نسبة الهيموجلوبين بين ٧ و ١٢ديسيليتر/جرام). من مجموع ٢٧٣ عزلة تم تعريف ٧٤ % منها على أنها ايروموناس. توزعت بين أنواع ثلاثة هى؛ هيدروفيلا ٤٥ %، بنكتاتا ٣٤ % و سالمونيسيدا ٢١ %. و كان انتشارها بين الحالات المرضية المختلفة مؤكدا للدور الذى يلعبه نوع هيدروفيلا فى حالات الفشل الكلوى (٨٣ %) و حالات التهاب الكلى (٦٧ %)، بينما ساد نوع سالمونيسيدا في حالات التهاب المهبل تحت الدراسة. تم اختبار ٩ عزلات مختارة لمقاومة خمسة من أكثر المضادات الحيوية شيوعاً في الاستخدام وهى: الامبسيلين والكلورامفينكول و التتراسيكلين والستربتوميسين وفنكومايسين. وجد إن كل العزلات حساسة لكل المضادات الحيوية ما عدا عزلتين من اصل بشرى كانت مقاومة للامبسيلين وفنكومايسين. احتوت كل منها على بلازميد واحد تراوحت احجامها الجزيئيه ما بين ٢٤ الى ٣٢٤ (كيلو قاعدة مزدوجه). دلت نتائج تجارب الشفاء من البلازميدات و اعادة تقدير " التركيز الأدنى المثبط" من المضاد الأحيائى امبيسيللين على الأرتباط الشديد بين مقاومة هذه العزلات لهذا المضاد الحيوى و بين وجود البلازميدات. اكدت هذه النتائج تجارب قياس" التركيزالأدنى المثبط" للمضاد الأحيائى ذاته على خلايا من سلالة ايشريشيا كولاى (D H 1) منقول لها هذه البلازميدات. استنتجت هذه الدّراسةُ توزيع ودرجةِ تدخل عالي لجنس بكتيريا ايروموناس في مصادر الطّعام المختلفةِ والأمراض المختلفة البشرية في محافظة الإسماعيلية–مصر.