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PLASMID PROFILE, ANTIMICROBIALS MINIMAL INHIBITORY CONCENTRATION AND *IN-VIVO* SENSITIVITY OF *SALMONELLA TYPHIMURIUM* AND *SALMONELLA COELN* ISOLATED FROM PIGEONS IN UPPER EGYPT

(With 3 Tables and 1 Figure)

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توصيف البلازميد وأقل تركيز مثبط من المضادات الحيوية وكفاءتها داخل الجسم للسالمونيلا تيفيموريم والسالمونيلا كولين المعزولة من الحمام في صعيد مصر

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في دراسة على معزولات السالمونيلا تيفيموريم والسالمونيلا كولين ثبت عزل البلازميدات متشابهة في العدد والوزن الجزيئى بين العترات الموجودة فى نفس النوع. كان الوزن الجزيئى للبلازميدات المعزولة من السالمونيلا تيفيموريم هو ٤٥ و ٦٩ كيلوبيز بينما في عترات السالمونيلا كولين كان الوزن الجزيئى للبلازميدات المعزولة هو ٥٧ و ٧٢ كيلوبيز. كانت العلاقة الخاصة بمقاومة المضادات الحيوية لعترات السالمونيلا كولين فردية ضد السلفاكينوكسالين بينما كانت العلاقة مزدوجة بالنسبة لعترات السالمونيلا تيفيموريم ضد السلفاكينوكسالين والإريثرومايسين. وجد أن هناك علاقة متعددة بمقاومة المضادات الحيوية (الكوليستين، الفليموكوين، نالادكسيك أسد، النيومايسين، الإريثرومايسين، الكلورتتراسيكلين، الأوكستتراسيكلين و السلفاكينوكسالين) وذلك للسالمونيلا تيفيموريم أما بالنسبة للسالمونيلا كولين فكانت ضد (الكوليستين، الفليموكوين، نالادكسيك أسد، النيومايسين، والسلفاكينوكسالين). كانت هناك حساسية كاملة من كلا نوعى السالمونيلا إلى الإنروفلوكساسين والأموكسيسيللين. وفى داخل جسم الطائر وجد أن الإنروفلوكساسين أوقف إفراز ميكروبات السالمونيلا تيفيموريم وقلل نموها داخل الأمعاء بعد اليوم الثالث من العلاج وحتى آخر التجربة، في حين عند إستخدام الأموكسيسيللين ثبت إفراز الميكروب مرة أخرى عند اليوم السادس والعشرين من توقف العلاج.

SUMMARY

Salmonella typhimurium and *Salmonella coeln* isolates obtained from pigeons in Upper Egypt revealed detection of low copy number plasmids which were similar in number and molecular weight among strains of the same serovar. Plasmids of molecular weights 69 and 45 Kb were isolated from *Salmonella typhimurium*, while 72 and 57 Kb were recovered from *Salmonella coeln*. A single pattern of antimicrobial resistance was recorded to sulphaquinoxaline among strains of *Salmonella coeln*, while double drug resistance pattern was common against sulphaquinoxaline and erythromycin among strains of *Salmonella typhimurium*. Multiple drug resistance was observed for both *Salmonella typhimurium* and *Salmonella coeln*. Complete sensitivity to enrofloxacin and amoxycillin was noticed by both serovars. *In-vivo* sensitivity of *Salmonella typhimurium* revealed that enrofloxacin prevented shedding of micro-organisms and minimized the intestinal colonization after the 3rd day of treatment till the end of the trial, followed by amoxycillin which showed reshedding of micro-organisms at 26th day from stopping of the treatment.

Key words: *Plasmid profile, Antimicrobials, salmonella typhimurim, Salmonella coeln, pigeons.*

INTRODUCTION

Salmonellosis constitutes one of the important diseases infecting wide spectrum of hosts such as wild and domestic animals, human and birds (Walton, 1983; Blood et al., 1990; Nagarja and Ekperigin, 1998).

One of the most important sources of protein in Egypt is pigeons. Another uses of pigeons in races and shows make them important. Salmonellosis causes up to 20%-30% mortalities in young ages of pigeons as well as adult ages. Moreover it is debilitating factor, reduce the fertility and hatchability (Tudor, 1991). It is of public health significance causing food poisoning in human (Pontello et al., 1982 and Lax et al., 1995). In Egypt few trials had been done to cover the paratyphoid infection in pigeons (Emmel, 1929; Khalifa, 1935; Ahmed and El-Sisi, 1965; El-Agroudi and Sadek, 1966; El-shater, 1979).

The aim of the present work is plasmid profile analysis of the isolated bacteria, antibiogram to certain antibiotics as well as

determination of minimal inhibitory concentration (MIC) of isolated motile salmonellae against a selected antimicrobials. A Furthermore conduction of *in-vivo* experiment using the most effective antibiotics in MIC and *in-vitro* sensitivity.

MATERIALS and METHODS

Bacterial strains:

Ten selected isolates of *Salmonella* were used, seven of them were *Salmonella typhimurium* and three were *Salmonella coeln.*

Determination of (MIC):

MICs of antimicrobial agents were determined by agar dilution method according to (Mitsuhasi et al., 1981). Ten antibiotics supplied by Amoun Industrial Comp., Egypt were used in this study. These antibiotics included Colistin sulphate (CT), Amoxycillin (AML), Flumequine (FL), Nalidixic acid (NA), Neomycin (N), Enrofloxacin (ENR), Erythromycin (E), Chlortetracycline (CTC), Oxytetracycline (OTC) and Sulphaquinoxaline (SQ). The stock solutions of antimicrobials were made in sterile distilled water except for oxytetracycline and chlortetracycline were done in ethanol.

Serial two fold dilutions of a forementioned antibiotics were done in the range of 0.2-100 µg/ml according to individual MIC break point of each antimicrobial agent.

Efficacy of enrofloxacin and amoxycillin in control of salmonella infection in pigeon:

In a screening experiment using disk diffusion method for determination of sensitivity of *Salmonella typhimurium* to several antimicrobial agents, results were high sensitivity to enrofloxacin followed by amoxycillin. Those antibiotics were used for disease control after experimental infection.

Thirty, 45-days-old squabs were divided into 4 groups, ten squabs each. The first two groups were used to study the efficacy of enrofloxacin and amoxycillin, while the third one kept as infected non-treated control. All groups were challenged with $4x10^8$ CFU of *Salmonella. typhimurium.* Antibiotics were used for treatment following appearance of clinical signs directly. The antibiotics were applied in drinking water for 5 successive days in a dose of 100 and 200 mg/liter of enrofloxacin and amoxycillin, respectively. All squabs were examined

for mortality rate, bacterial shedding, protection rate and total colony count of intestinal colonizing *Salmonella* per gram of intestinal contents.

Isolation of plasmid DNA:

Bacteria from nutrient agar (Oxoid) slope were plated out onto selective enrichment plating medium *Salmonella-Shigella* agar (Biolife) for 18-24 hours. A single colony was picked and inoculated into 10 ml of Luria-Bertini broth (LB-broth) and grown with shaking at 37°C overnight for 18 hours in shaking water bath. The cells were harvested by centrifugation for 5 minutes at 12.000 rpm. The bacterial pellet was used for plasmid isolation. Alkaline lysis method of (Brinboim and Doly, 1979) was carried out. The ethanol precipitated plasmid DNA was kept in Tris-EDTA buffer (PH 8.0) at -20°C for electrophoresis.

Agarose gel electrophoresis:

Electrophoresis was carried out in horizontal 0.7% agarose gel system (BioRad, Richmond, USA). The running buffer was GGB buffer (PH 8.3). The prepared plasmid DNA was treated by RNase enzyme and mixed with loading buffer, then inoculated to gel tray, the electric field used as 75 mA for 2-3 hours. The standard Marker was the isolated plasmids obtained from *E.coli*- V517 of molecular weight ranged from 1.4-35.8 Mda. The gel was stained by 0.5 µg/ml ethidium bromide solution for 20-30 minutes and washed by distilled water for 20 minutes and photographed by direct screen instant camera (Polaroid DS.34) under Ultraviolet transilluminator (TFX-20M, Vilber Lourmat -France). The molecular weights were determined by matching the electrophoretic mobility of both marker and isolated plasmid DNA.

RESULTS

MIC:

Results are shown in Table 1. *Salmonella* isolates used in this study were completely sensitive to Enrofloxacin and Amoxycillin with MIC value ranged from 0.78-1.56 and 0.39-0.78 µg/ml respectively. Complete resistance were observed for sulphaquinoxaline. 7/10 of tested strains resist the action of erythromycin, 2/10 were resistant to colistin sulphate, flumequine, nalidixic acid and neomycin, while only 1/10 was resistant to oxytetracycline and chlortetracycline.

Efficacy of Enrofloxacin and Amoxycillin in control of Salmonella infection in pigeons:

Enrofloxacin and amoxycillin treatment of experimentally infected pigeons showed lower number of excreted *Salmonella* than

control birds. In case of Enrofloxacin-treated group, there were disappearance of Salmonella excretion in the droppings after 3rd day post treatment, while in the amoxycillin-treated group the excretion of Salmonella disappeared after the 3rd day of treatment till the period of 15 days but start again 11 days after stopping the treatment. The isolation rate of *Salmonella* from intestinal culture of infected squabs was 100% in control group followed by 30% and 20% in amoxycillin-and enrofloxacin-treated groups.

Salmonella typhimurium challenged control group had mean $\log_{10}$ of 5.36±1.05 *S.typhimurium* /g of intestinal content, whereas the amoxycillin-treated group was 1.58±0.35, while in enrofloxacin-treated group was 1.01±0.56 *S. typhimurium*/g of intestinal content. Results are illustrated in Table 2.

Plasmid profiling:

The plasmid DNA isolated from *Salmonella typhimurium* and *salmonella coeln* were curable and of low copy number. All of the tested seven isolates of *Salmonella typhimurium* possessed two plasmids of 69 and 45 Kb, while in case of *Salmonella coeln* two plasmids were recovered in the tested three isolates of molecular weight 72 and 57 kb. Results are shown in Fig.1. A 100% resistance to Erythromycin and sulphaquinoxaline were recorded in case of *Salmonella typhimurium*. The resistance patterns to the tested antibiotics and its relationship with existence of plasmid DNA of both *Salmonella typhimurium* and *Salmonella coeln* were listed in Table 3.

DISCUSSION

Although paratyphoid infection is well known since the 19th century as investigated by Moore (1985), the disease until now is still as one of the most important diseases and of great economic and zoonotic importance in veterinary field. The disease is responsible for severe losses due to lowering of fertility and hatchability as well as high moralities in young ages.

In present study we tried the isolation of plasmid DNA from pigeon isolates (*Salmonella typhimurium* and *Salmonella coeln*) as well as MIC determination and *in-vivo* sensitivity of selected antibiotics (Enrofloxacin and Amoxycillin).

Bacterial plasmids are extrachromosomal DNA known to be code for toxin production, adhesiveness, antibiotic resistance and serum

resistance (Baroun and Ou, 1991; Riikonen et al., 1992 and Lax et al., 1995).

Most of wild-type bacteria seem to contain plasmids of different size and number.

The plasmid pattern of bacterial strain may be specific during certain interval and in a limited area. This property has rendered the determination of such pattern a potentially powerful tool for epidemiologic studies (Elwell et al., 1978).

The examined seven strains belonging to *Salmonella typhimurium* showed that they bear tow plasmids of molecular weights 64 and 45 Kb, but the three strains of *Salmonella coeln* found to bear tow plasmids of molecular weights 72 and 57 Kb. From the previously mentioned results, plasmid profile is clearly similar among strains of the same serotype. Similar findings were obtained by Felix et al. (1983); Threlfall et al. (1989); Odongo et al. (1990), and Daniel et al. (1992) who noticed the similarity of plasmid profiles of strains belonging to the same serovar, as well as they mentioned that the plasmid profiling has been a useful tool for subdivision and a simple, sensitive assay to provide a limited strain differentiation for laboratories.

The data presented in this study showed a homology of the molecular-weight plasmids inside the same serotype which support the hypothesis of the evolution and spread of a single clone of *Salmonella typhimurium* and *Salmonella coeln* in Asssiut province. Our result may be supported by the findings of Brown et al. (1986), Baggesen et al. (1992) and Christensen et al. (1994) who recorded the presence of the same clonal lines due to isolation of the same molecular weight plasmids among the same serotypes.

According to the present results it could be concluded that the infection of pigeons is ascribed to a single infection due to presence of the same molecular weights of plasmid of the same serovar.

The plasmid profiles of the isolated *Salmonella typhimurium* strains were of large size, and of low copy number with molecular weights of 64 and 45 Kb. These results supported by Felix et al. (1983), Susan et al. (1988), and Purushothaman et al. (1996) who found that 45.7 to 74 Kb molecular weight plasmids were present in *Salmonella typhimurium* isolates. On the other hand Threlfall et al. (1994) recorded that the dominant clonal lines of *almonella typhimurium* were associated with plasmid profiles that have 94 Kb. This difference may be due to gaining or loosing plasmid from the dominant clonal lines of plasmid

profiles which may represent sublines from developed dominant lines Baggesen et al. (1992).

Concerning the antibiotic resistance, the present results revealed that among the seven isolates of *Salmonella typhimurium*, one multiple resistance pattern was observed against colistin sulphate, flumequine, nalidixic acid, neomycin, oxytetracycline, chlortetracycline, and sulphaquinoxaline and another multiple resistance pattern among the three strains of *Salmonella coeln* against colistin sulphate, flumequine, nalidixic acid, neomycin, and sulphaquinoxaline. Six out of 7 isolates of *Salmonella typhimurium* were resistant against sulphaquinoxaline and erythromycin. On the other hand, tow out of 3 strains of *Salmonella coeln* showed a single resistance against sulphaquinoxaline. The present results supported by the findings of Felix et al. (1983) who found only resistance against sulphonaimdes, and Purushathaman et al. (1996) who found 62% of *Salmonella typhimurium* isolates were resistant to erythromycin. In contrast to our results, Niida et al. (1983) mentioned a resistance against streptomycin, tetracycline, sulphonamides, chloramphenicol, kanamycin, and ampicillin, but most of cultures were resistant against tetracycline and sulfonamides. In addition, Verma and Gupta (1994) revealed a multiple drug resistance against ampicillin, chloramphenicol, kanamycin, streptomycin, trimethoprim and tetracycline.

The relation between possession of plasmid DNA of the tested *Salmonella* isolates and their antimicrobial resistance patterns showed that all isolates of *Salmonella typhimurium* (100%) had plasmids and drug resistance. Most of *Salmonella typhimurium* strains possessed a homology against sulphaquinoxaline and erythromycin resistance. On the other hand, all strains of *Salmonella coeln* possessed plasmids and drug resistance, two strains out of the 3 had resistance against sulphaquinoxaline. Consequently we can deduce that the plasmids may play an important role in the observed drug resistance among *Salmonella* isolates. Our present results support the previous findings reported by Poppe and Gyles (1987) and Odongo et al. (1990) who noted that the large plasmids (> 30 Mda) were demonstrated in antibiotic resistance *Salmonella* cultures, and also Felix et al. (1983) and Jack and Hirsh (1985) who attributed the multiple drug resistance pattern to be usually associated with carriage of plasmids.

The authors concluded in the present study that the plasmid DNA of *Salmonella* isolates is large, and of low copy number. The molecular

weights were 64; 45 Kb for *Salmonella typhimurium* and 72; 57 Kb for *Salmonella coeln* strains. Plasmid profiling of the examined strains strongly indicated that we were dealing with persistent infection which directed us to made an improvement in loft construction, sanitation and disinfection procedures in addition to biosecurity in general to prevent reinfection.

The antimicrobial resistance of *Salmonella* isolates was common and the plasmids may play a role in this resistance, but further studies are required with aid of transformation and transconjugation to confirm the relation between plasmid and antimicrobial resistance.

Salmonellosis in pigeons is difficult to treat and to eradicate especially in lofts, because birds remain chronically infected and intermittently excrete the bacterium (Devriese, 1986). Antibiotic treatment has not been evaluated in-vivo experiments with adequate controls. Under field conditions, controlled trials are almost impossible to perform. Therefore, we decided to compare different antibiotic treatments in pigeons infected experimentally with *Salmonella typhimurium*.

It is clear that, there is a decrease in the *Salmonella* excreting pigeons in all treated groups compared with control one. The enrofloxacin proved to be highly effective in treating pigeon infected group. The clinical signs subsided rapidly, shedding of the organism by infected pigeons was negative after the 5th day of treatment till the end of the trial. On the other hand, amoxycillin also effective in treating the other group of infected pigeons but less than that obtained following the treatment with enrofloxacin. Amoxycillin-treated group showed negative shedding of the organisms after 5^{th} day of treatment until the 28^{th} day post-treatment but shedding of the micro-organisms had been started to appear at the 26^{th} day from stopping of the drug. Concerning the counts of *Salmonella*/gram intestinal contents, the enrofloxacin had a highly significant decrease in the mean number of *Salmonella* which followed by amoxycillin. Results also revealed that enrofloxacin-treated group had the highest protection and the lowest mortality rates followed by amoxycillin.

The results were parallel to those of the *in-vitro* sensitivity. This similarity between the *in-vivo* and *in-vitro* sensitivity, also reported by Smith (1955).

Our findings were similar to those reported by Goosens et al. (1985) who found that the minimum inhibitory concentration (MIC) of

fluoroquinolone antibiotics for *Salmonella* in low and high therapeutic concentrations are achieved in serum, tissues, and faeces after oral administration. Fluoroquinolone excretion in man persists several days after cessation of therapy, thereby effectively prolonging the duration of antibacterial activity. A similar effect was not seen following treatment with amoxycillin. The elimination half life and the tissue distribution pattern is comparable to the findings in poultry but tissue levels are nearly 3-4 times higher (Dorrestein, 1986). Dorrestein and Verburg (1988) mentioned that the enrofloxacin is absorbed readily after oral application and give high serum level in pigeons compared to serum levels in chicken serum. In France, Guillot (1989) studied the *in-vivo* activity of enrofloxacin against *Salmonella* in the gut of birds and observed that enrofloxacin seems to have a good efficiency against the intestinal carriage of salmonellae, nearly similar results was also reported by Schmahl, (1993).

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Table 1. Susceptibility of 10 pigeon isolates of *Salmonella typhimurium* and *Salmonella coeln* to different antibacterial agents:

Antibacterial agents	No. of isolates with MIC (ug/ml) of										MIC (ug/ml) break point of resistance	Number of resistant strains
	0.2	0.39	0.78	1.56	3.12	6.25	12.5	25	50	100		
CT	-	-	7	1	-	2	-	-	-	-	≥ 6.25	2
FL	-	7	-	-	1	2	-	-	-	-	≥ 6.25	2
NA	-	-	8	-	-	2	-	-	-	-	≥ 6.25	2
N	-	-	-	6	2	-	2	-	-	-	≥ 12.5	2
ENR	-	-	9	1	-	-	-	-	-	-	≥ 12.5	-
E	-	-	2	-	1	7	-	-	-	-	≥ 6.25	7
AML	-	9	1	-	-	-	-	-	-	-	≥ 6.25	-
CTC	-	-	3	4	-	1	1	-	1	-	≥ 50	1
OTC	-	-	2	3	2	2	-	-	1	-	≥ 50	1
SQ	-	-	-	-	-	-	-	-	-	-	≥ 100	10

N.B. CT, Colistin sulphate; FL, Flumequine; NA, Nalidixic acid; N, Neomycin; Enr, Enrofloxacin; E, Erythromycin; AML, Amoxycillin; CTC, chlortetracycline; OTC, Oxytetracycline; SQ, Sulphaquinoxaline.

Table 2. Effect of providing different antibiotics on *Salmonella* colonization and protection rate in squabs exposed to *Salmonella typhimurium*.

Treated group rate	*S.typhimurium* challenge dose	Log_{10} *Salmonella*/g intestinal contents	*Salmonella* culture Positive/total (%)	Number of protected birds	Protection
Control	$4X10^8$	5.36 ± 1.05^B	10/10 (100)	0/10	0%
Enrofloxacin	$4X10^8$	1.01 ± 0.56^b	3/10 (30)	9/10	90%
Amoxycillin	$4X10^8$	1.58 ± 0.35^b	3/10 (30)	7/10	70%

N.B.
B = Mean ± SD values followed by lower case superscripts are significantly different from controls: a = $P < 0.05$, b = $P < 0.01$.

Table 3. Relationship of possession of plasmid DNA and Drug resistance patterns of *S. typhimurium and S.coeln*:

Isolate Number	*Salmonella* serotype	Plasmid profiles		Antibiogram
		Number of plasmids	Molecular size of plasmid DNA (KB)	
S.243	*typhimurium*	2	69; 45	E, SQ
S.249	*typhimurium*	2	69; 45	CT, FL, NA, N, E. CTC, OTC, SQ
S.250	*typhimurium*	2	69; 45	E, SQ
S.251	*typhimurium*	2	69; 45	E, SQ
S.252	*typhimurium*	2	69; 45	E, SQ
S.255	*typhimurium*	2	69; 45	E, SQ
S.289	*typhimurium*	2	69; 45	E, SQ
S.920	*coeln*	2	72; 57	CT, FL, NA, N, SQ
S.939	*coeln*	2	72; 57	SQ
S.950	*coeln*	2	72; 57	SQ

N.B.
E, Erythromycin; SQ, Sulphaquinoxaline; CT, Colistin sulphate; FL, Flumequine; NA, Nalidixic acid; N, Neomycin; CTC, Chlortetracycline; OTC, Oxytetracycline.

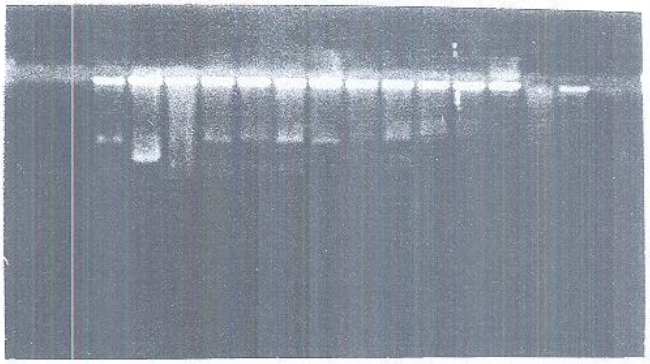

Figure 1. M: Plasmid molecular size marker *E.coli* V517 (35.8 to 1.4 Mdal), Lane 1 to lane 7 *Salmonella typhimurium* with the 69. 45 kb profile: lane8 to 10 *Salmonella coeln* with the72. 57 profile.