ELECTROPHORETIC CHANGES IN ESCHERICHIA COLI AND CLOSTREIDIUM PERFRINGENS RECOVERED FROM ENTERITIS IN QUAILS AFTER EXPOSURE TO ENROFLOXACIN

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ABSTRACT

E. coli and C. perfringens were isolated from the intestinal contents of quails suffering from enteritis at different incidences either alone or concurrently mixed together. C. perfringens types A and D were identified and type D was the most predominant. E. coli isolates were sensitive to enrofloxacin, ceflifur, chloramphenicol, flumequine and spectinomycin. Meanwhile, C. perfringens isolates were sensitive to ampicillin, penicillin-G, lincomycin, enrofloxacin and flumequine. MIC of enrofloxacin to E. coli was 80 µg/ml and its MBC to C. perfringens type D was 2.5 µg/ml. Electrophoretic analysis of E. coli and C. perfringens type D strains post-exposure to small doses of enrofloxacin showed marked differences in the number of the obtained bands, molecular weight of each band and amount of the band in comparison to the obtained results before their exposure to enrofloxacin. These results explain the appearance of mutants post-exposure to mild doses of antibiotics. These mutants differ in structure than their parents and have marked affinity for antibiotic resistance.

INTRODUCTION

E. coli was the main pathogen in quails. The infection was acute in 7-day old birds with rapid death and few lesions. In 14-week old birds, the lesions seen were typical to colibacillosis in fowls; most birds showed air sacculitis, fibrinous pericarditis and perihepatitis. It was suggested that quails may play an important role in the spread of E. coli in fowls, when they are in contact **(Reddy and Koteeswaran, 1994)**. Moreover, E. coli caused coligranulomatosis in 8 - 12 month old Coturnix quails **(Silva et al., 1989)**. It is concluded that the stress factors such as intensive methods of housing lead to a high possibility of E. coli infections in a farm of 5000 Japanese quails, Coturnix Coturnix Japonica **(Franchesi et al., 1995)**.

C. perfringens was recovered from dead quails with a history of necrotic and ulcerative enteritis. C. perfringens type A was highly pathogenic for young quails than old ones **(El-Bardisy, 1999)**. Oral administration of C. perfringens type C in quails showed that 2 out of 10 birds developed clinical symptoms of necrotic enteritis after 3 - 6 days. Slaughtered quails at 4 weeks post-infection showed lesions generally similar to that in naturally infected chicks **(Cygan and Nowak, 1974)**.

As the result of administration of low doses of antibiotics for the treatment of infected birds, new strains of the same organisms were developed carrying genes resistant for these antibiotics **(LiHong et al., 1999; and Daly and Fanning, 2000)**.

Thus, the aim of this study was to evaluate the electrophoretic changes, which occur in E. coli and C. perfringens exposed to low levels of enrofloxacin and recovered from diarrhoeic quails.

MATERIAL AND METHODS

A total of 50 intestinal tracts were collected from 3-weeks old quails suffering from enteritis at private farms in Kafr El-Sheikh Governorate and examined bacteriologically for the presence of E. coli and C. perfringens.

Isolation of E. coli was done by cultivation aerobically onto MacConkey's agar and eosin methylene blue agar plates at 37°C for 24 - 48 hours. Suspected colonies were identified according to **Koneman et al. (1992) and Quinn et al. (1994)**. Dermoreactive ones from the identified E. coli isolates were detected as described by **Abd El-Gaber et al. (2001)**.

C. perfringens was isolated from the anaeroble growth of samples in cooked meat broth followed by subculturing onto 200µg/ml neomycin sulphate sheep blood agar plates. The inoculated tubes and plates were incubated anaerobically at 37°C for 24 hours. The obtained isolates were identified as described by **Koneman et al. (1992)**. Typing of C. perfringens isolates was made using dermo-necrotic test in guinea-pigs **(Sterne and Batty, 1975)**.

Antibiogram of E. coli and C. perfringens isolates were studied as described by **Koneman et al. (1992) and Quinn et al. (1994)**. Bacterial species whose isolates exceeded 50% susceptibility to antibiogram was recorded as sensitive to it (S), those less than 50% susceptibility was recorded as resistant (R), but the species whose isolates produced 50% moderate susceptibility was recorded moderate (SR) as described by **Abd El-Gaber and Rezeka (1999)**.

One strain represented each of E. coli and C. perfringens type D were subjected to MIC and MBC as well as electrophoretic studies.

Enrofloxacin was chosen in this study, where most of isolates of both E. coli and C. perfringens were sensitive (S) to it. Different dilutions were prepared as follows:

Serial 2-fold dilutions of enrofloxacin were prepared in brain-heart infusion broth starting from a concentration 100 mg/ml by mixing 1 mg enrofloxacin in the broth till 10 ml. 2 ml from the 1st dilution was used in this experiment where 1 ml was transferred from it into the 2nd tube containing 1 ml broth to obtain 50 µg/ml concentration and so on were the concentration of antibiotic in the 3rd tube was 25 µg/ml, the 4th tube was 12.5 µg/ml, 5th tube was 6.25 µg/ml, 6th tube was 3.125 µg/ml, 7th tube was 1.562 µg/ml, 8th tube was 0.781 µg/ml, 9th tube was 0.39 µg/ml and 10th tube was 0.195 µg/ml.

0.25 ml of standard bacterial suspension prepared from the tested organism was added into each tube where the final dilutions were 80, 40, 20, 10, 5, 2.5, 1.25, 0.625, 0.3125 and 0.15625 µg/ml.

Control tube was made by adding 0.25 ml of the tested organism into a tube containing 1ml broth alone without antibiotic.

The tubes were incubated either aerobically for E. coli or anaerobically for C. perfringens at 37°C for 24 hours. The last tube showing no bacterial growth (no turbidity) is the end point (MIC). The tube of end point was centrifuged and discard the supernatant. Subculture from the sediment onto the surface of solid media was done to determine MBC. **(Quinn et al., 1994)**.

Bacterial count was made from the different dilutions showing bacterial growth onto the surface of MacConkey's agar plates for E. coli and sheep blood agar plates without antibiotic for C. perfringens. 0.1 ml from each dilution was spread onto the surface of solid media and 2 plates were used for each dilution. The inoculated plates were incubated either aerobically for E. coli or anaerobically for C. perfringens. The count in each dilution was calculated as follows:

Bacterial count in the dilution= (Count in the first plate + Count in the second plate)/2 X 10.

The isolated bacteria from the first and/or second dilution showing bacterial growth and following end point were subjected to electrophoretic study as described by Abd El-Gaber et al. (2001). Molecular size marker of 6 different molecular weights and amounts 216 (10.097), 199 (35.182), 167 (11.849), 99 (16.685), 66 (15.882) and 29 (10.231) was used in this study.

RESULTS AND DISCUSSION

As shown in table (1), dermo-necrotic E. coli was isolated from the intestinal contents of 18.00% quails suffering from enteritis as well as from 6.00% as a mixed infection with C. perfringens. Meanwhile, C. perfringens was isolated alone from 16.00% infected birds. **Silva et al.**

(1989) recorded the occurrence of coligranulomatosis in a Brazilian flock of 8 - 12 month old Coturnix quails causing 15.00% mortality. Lesions were located on the mesentery, intestines, gizzard, heart, oviducts, ovaries, and liver. **Reddy and Koteeswaran (1994)** recorded that the infection of quail chicks was acute in 7-day old birds with rapid death and few lesions. In 4-week old birds, the lesions seen were typical of colibacillosis in fowls. **El-Bardisy (1999)** isolated C. perfringens from 35.00% of quails suffering from necrotic and/or ulcerative enteritis. The isolation was high from the intestinal contents (40.00%). C. perfringens type A was the most predominant type and highly pathogenic for young quails than old ones.

In Tables (2,3,4 & 5), E. coli isolates were sensitive to enrofloxacin, ceftifur, chloramphenicol, flumequine and spectinomycin. Meanwhile, C. perfringens isolates were sensitive to ampicillin, penicillin-G, lincomycin, enrofloxacin and flumequine. Zone diameter of growth inhibition of enrofloxacin to the chosen isolates of E. coli and C. perfringens type D was 27 mm and 22 mm respectively. MIC of enrofloxacin to E. coli was 80 mg/ml and its MBC to C. perfringens was 2.5mg/ml. **Walser et al. (1993)** recorded that MIC of E.coli (200 strains) isolated from milk of mastitic cows was ranged from 0.03 - 1.0 with median value 0.06 and MIC 90% 0.14 mg/ml. **Ziv et al (1998)** found that norfloxacin, enrofloxacin and cefotaxime were the best in vitro activity against E. coli with MIC 90 values of $\leq$ 0.25 mg/ml. **Liang et al (1998)** recorded that MIC of enrofloxacin against E. coli O78 was 3.906 X 10-3 mg/ml and the inhibition zone in K-B disk diffusion test was 30 $\pm$ 2 mm. **Sayed et al. (1998)** recorded the susceptibility of both Salmonella enek and E. coli to enrofloxacin, flumequine, chlortetracycline, oxytetracycline, erythromycin and neomycin. MIC values for these drugs varied from 0.2 to 3.13 mg/ml. **Soliman (2000)** found that MIC of enrofloxacin against E. coli isolated from broilers was 0.064 mg/ml. On the other hand, **Kim et al. (1997)** recorded that at the MIC of enrofloxacin or colistin, both antibacterial agents showed the highest killing rates during 2 - 4 hours against Gram-negative bacteria such as E. coli K88ab, P. multocida type A, and Bordetella bronchiseptica but allowed the re-growth of the same pathogens afterwards. However, the combination of the two antibacterial agents at a fourth MIC resulted in a high killing rate without bacterial re-growth during 24 hours.

As shown in tables (6 and 7) and figure (1), electrophoretic analysis of E. coli and C. perfringens type D strains exposed to small doses of enrofloxacin in vitro showed differences in the number and molecular weight of bands and in the amount of each band in both organisms in comparison to the obtained bands before their exposure to enrofloxacin. Furthermore, E. coli lost 5 bands and C. perfringens type D lost 4 bands. These results explain the appearance of mutants, which differ completely in their structure than their parents as well as their affinity to resist the antibiotics previously, primed to them.

Table (1): Incidence of E. coli (dermo-necrotic one)and C. perfringens in quails suffering from enteritis.

Exam-ined birds	E. coli		C. perfringens						Mixed infection			
	No.	%	Type A		Type D		Total		E.coli & C.perf. type A		E.coli & C.perf. type D	
			No.	%	No.	%	No.	%	No.	%	No.	%
50	9	18	2	4	6	12	8	16	1	2	3	6

Table (2): Susceptibility of the obtained isolates of E. coli and C. perfringens to different chemotheraprutic agents.

Chemotherapeutic agent	E. coli (13)			C. perfringens (12)		
	No.	%	AA	No.	%	AA
Ampicillin	4	30.76	R	10	83.33	S
Ceflifur	8	61.53	S	4	33.33	R
Chloramphenicol	7	53.84	S	5	41.66	R
Colistin sulphate	3	23.07	R	4	33.33	R
Doxycillin Hcl	1	7.69	R	5	41.66	R
Enrofloxacin	9	69.23	S	7	58.33	S
Erythromycin	1	7.69	R	1	8.33	R
Flumequine	7	53.84	S	6	50.00	S
Gentamicin	3	23.07	R	2	16.66	R
Lincomycin	2	15.38	R	8	66.66	S
Nalidixic acid	4	30.76	R	1	8.33	R
Neomycin sulphate	3	23.07	R	1	8.33	R
Oxolinic acid	4	30.76	R	1	8.33	R
Oxytetracycline	2	15.38	R	1	8.33	R
Penicillin-G	1	7.69	R	9	75.00	S
Spectinomycin	7	53.84	S	5	41.66	R
Streptomycin	3	23.07	R	1	8.33	R
Sulphamethoxazole +trimethoprim	5	38.46	R	1	8.33	R

No.: Number of sensitive isolates.

%: Percentage of sensitive isolates in relation to the total species isolates.

AA: Antibiogram activity.

Table (3): Zone diameter of growth inhibition of enrofloxacin to the chosen isolates of E. coli and C. perfringens type D for in vitro MIC and MBC studies and for electrophoretic studies.

Organism	Zone diameter of growth inhibition
E. coli	27 mm
C. perfringens type D	22 mm

Table (4): Count of E. coli and C. perfringens type D strains post-exposure to different dilutions of enrofloxacin in comparison to bacterial growth in the form of turbidity in liquid media.

Enrofloxacin dilutions(µg/ml)	E. coli		C. perfringens type D	
	Turbidity	Count	Turbidity	Count
80	-	2	-	-
40	+	65	-	-
20	++	105	-	-
10	+++	UHG	-	-
5	+++	UHG	-	-
2.5	+++	UHG	-	-
1.25	+++	UHG	+	10
0.625	+++	UHG	++	37
0.3125	+++	UHG	+++	95
0.1625	+++	UHG	+++	UHG

- : No bacterial growth and no bacterial count.
+ : Weak bacterial growth.
++ : Moderate bacterial growth.
+++ : Heavy bacterial growth.
UHG: Uncountable heavy growth.

Table (5): MIC or MBC of enrofloxacin to E. coli and C. perfringens type D strains.

Organism	MIC	MBC
E.coli	80 µg/ml	-
C. perfringens type D	-	2.5 µg/ml

Table (6): Electrophoretic analysis of E. coli before and after exposure to enrofloxacin.

Lanes: Bands	Marker		E. coli			
	M.W.	Amount	Before exposure		After exposure	
			M.W.	Amount	M.W.	Amount
1	216	10.097	213.39	6.3452	212.42	1.6797
2			210.18	1.9551	210.18	2.7411
3			204.51	7.0854	204.82	15.690
4	199	35.182	199	7.7301		
5					187.50	9.7686
6			176.66	2.0723		
7	167	11.849	165.81	13.437		
8			145.75	5.0258	145.75	8.6774
9			129.97	7.3761		
10			119.27	6.6776		
11					112.62	6.9468
12	99	16.685	99	3.5852	99	6.3823
13			93.428	9.7637		
14			88.171	2.0357	88.171	6.7362
15			83.477	4.2366	83.746	6 1993
16			77.024	4.0034	77.024	6.2706
17			69.487	6.4406	69.711	10.037
18	66	15.882	66	2.1866		
19					55.991	3.3527
20			47.067	6.1562	47.499	1.3445
21			40.295	0.92326	40.295	10.361
22			34.184	1.9974	34.184	3.7297
23	29	10.231	29	0.86311		
Sum		99.926		99.896		99.917
In lane		100		100		100

Table (7): Electrophoretic analysis of C. perfringens type D before and after exposure to enrofloxacin.

Lanes: Bands	Marker		C. perfringens type D			
	M.W.	Amount	Before exposure		After exposure	
			M.W.	Amount	M.W.	Amount
1	216	10.097	216	2.8337	216	3.0647
2			210.50	10.696	210.18	13.432
3			205.13	16.411	204.51	6.6864
4	199	35.182	199	2.4126	199	2.6355
5			187.50	3.3603	187.50	6.7902
6			176.66	8.7695		
7	167	11.849	165.81	2.8694		
8			145.75	1.8652	145.75	4.1105
9			128.12	3.4389	128.12	5.5132
10			112.62	2.2410		
11	99	16.685	99	2.5433	99	14.20
12			93.428	8.9378		
13			88.171	2.9112	88.171	3.6983
14			83.208	12.638	83.208	11.912
15			79.799	2.7305	79.033	9.9522
16			73.159	3.1508	71.529	0.69609
17	66	15.882	65.40	5.9718	66	9.9584
18			40.295	2.1471	40.295	3.1416
19			34.184	1.9339	34.184	1.3194
20	29	10.231	29	2.0971	29	2.7992
Sum		99.926		99.959		99.910
In lane		100		100		100

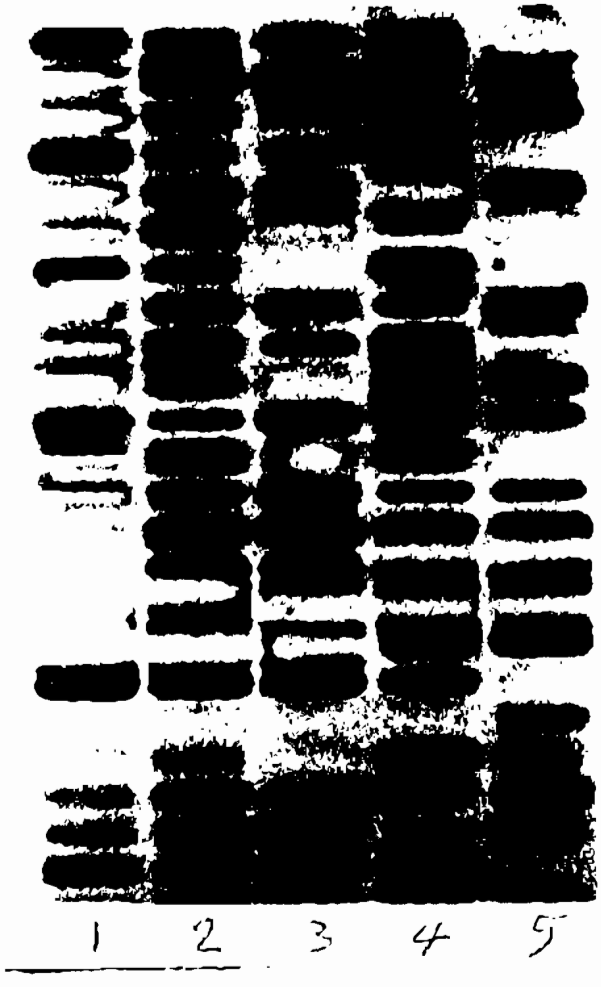

Fig. (1) : Electrophoretic analysis of E. coli and C. perfringens type D strains before and after exposure to enrofloxacin.
1: Marker
2: E. coli before exposure to enrofloxacin.
3: E. coli post-exposure to enrofloxacin.
4: C. perfringens type D before exposure to enrofloxacin.
5: C. perfringens type D post-exposure to enrofloxacin.

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الملخص العربى

التغيرات فى التحليل الكهربى لميكروبى الاشرشيا كولاى والكلوستريديم بيرفرنجنس المعزولان من حالات الالتهاب المعوي في السمان بعد التعرض للانروفلوكساسين

المشتركون فى البحث

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عزلت الاشرشيا كولاى والكلوستريديم بيرفرنجنس من محتويات الأمعاء من سمان يعانى من التهاب معوي بنسب مختلفة إما منفصلين أو مجتمعين معا. كلوستريديم بيرفرنجنس نوع أ، د هما النوعان المعزولان بحيث أن نوع د هو الأكثر عزلا. سجلت حساسية عترات الاشرشيا كولاى للانروفلوكساسين، سفليفيور، كلورامفنيكول، فلوميكوين، والاسبكتينومايسين على التوالى. في حين أن عترات الكلوستريديم بيرفرنجنس كانت حساسة للامبيسيللين، بنسيللين ج، لينكوميسين، انروفلوكساسين، والفلوميكوين على التوالى. وجد أن أقل تركيز للانروفلوكساسين مانع لنمو الاشرشيا كولاى هو ٨٠ ميكروجرام/ميليلتر وأقل تركيز قاتل للكلوستريديم بيرفرنجنس نوع د هو ٥و٢ ميكروجرام/ميليلتر. أظهر التحليل الكهربي لميكروبي الاشرشيا كولاى والكلوستريديم بيرفرنجنس نوع د بعد التعرض لجرعات صغيرة من الانروفلوكساسين اختلافات ملحوظة في عدد الشرائط والوزن الجزيئى لكل شريط وفى كمية الشريط مقارنة بالنتائج التى ظهرت قبل تعرضهم لهذا المضاد الحيوي. تبرر هذه النتائج ظهور عترات بكتيرية بعد التعرض لجرعات صغيرة من المضادات الحيوية. هذه العترات تختلف في تركيبها عن الآباء ولهم مقاومة ملحوظة للمضادات الحيوية.