Studies on some aerobic bacteria in ostriches in Egypt

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Abstract
A total of 220 samples from ostriches at different ages (100 from diseased ostriches and 120 from freshly dead) were collected from different ostriche's farms. All cases were subjected to post-mortem and bacteriological examinations. Most of the examined cases showed polybacterial infections. *E.coli, Protus mirabilis., Pasteurella haemolytica, Providenichia spp., Staphylococcus aureus, Salmonella typhimurium, Enterobacter cloacae, Aeromonas hydrophila* and *Shigella dysenteriae* were isolated from the examined cases with percentage of 37.8%, 27.7%, 12.1%, 5.4%, 5.04%, 4.2%, 3.3%, 2.5% and 1.6% respectively. *E. coli* isolates were serotyped as O2, O78, O114, O126 and untypable. Pathogenicity tests of the isolated microorganisms were studied in one day old chicks. Symptoms, mortalities and postmortem lesions were recorded and discussed in details. Fluoroquinolone antibiotics (ciprofloxacin, enrofloxacin and danofloxacin) were found to be highly effective against most of tested bacterial strains followed by gentamycin, colitin sulphate and sulphamethoxazole – trimethoprim. PCR technique was done on original samples and it appeared that PCR technique is more sensitive than the conventional microbiological technique (CMT).

Key words: *ostriches, serotypes, antibiotic sensitivity test .*

Introduction

Ostrich farming as a source for meat, skin and feathers, has been introduced into Egypt in recent years, the future success depends on its ability to supply high quality and safe product, the relatively little known about infectious disease, inadequate management practices and massive use of antibiotics without veterinary supervision are considered the major constrains to flock health and ostrich production **(Ali and Ibrahium, 2004** and **Abdel-Razik, 2007)**. Different bacterial agents incriminated in many diseased conditions of ostriches were associated with severe morbidity and mortality. Enteritis can be a primary infection with bacterial agents including *Salmonllae*, pathogenic strains of *E.coli*, *Klebsiella* and *Pseudomonase aeruginosa* **(Huchzermyer, 1999)**, causing diarrhea in young ostrich chicks **(Shapiro, 2001)**. Various serotypes of *E.coli* were isolated from ostrich which was accompanied with respiratory disorders **(Abdel-Razik, 2007)**, leg deformities and impaction of the proventriculus **(Terzich and Vanhooser, 1993)**, and late embryonic deaths during artificial inoculation of ostriches eggs **(Khafagy and Kamel, 2001)**. Moreover, *Pasteurella haemolytica* infection was isolated from many diseased cases of ostriches **(Nasef *et al*., 2003)**.

The aim of the present study was to detect the most prevalent pathogenic bacterial agents isolated from ostriches in Egypt at different ages with references to pathogenicity and antibiogram of the isolated organisms. Also, identification of salmonella spp. and staph spp. by the polymerase chain reaction (PCR) technique from the original samples broth.

Materials and Methods

Samples

A total of 220 samples of ostriches (100 samples from diseased ostriches and 120 samples from recently dead ones) were collected from different ostrich's farms at different localities in Egypt. Samples were collected aseptically and were representative for different ages. Collected samples included nasal and cloacal swabs from diseased ostriches, and organs (heart, liver, lung and bone marrow) from recently dead ostriches. Postmortem examination

was done for dead birds. Type and number of examined samples are shown in table (1).

Table (1). Type and number of examined ostriches samples

Types of samples	No. of samples
Cloacal swabs	50
Nasal swabs	50
Heart	30
Livers	30
lungs	30
Bone marrow	30
Total	220

Bacteriological examination

All collected samples were inoculated in buffer peptone water, brain heart infusion broth (Oxoid) and selenite-f-broth (Oxoid) and incubated at 37c◦ for 24 hours. Direct inoculation of different samples on sheep blood agar, nutrient agar and MacConkey agar plates then incubated all tubes and plates at 37c◦ for 24 hours. A loopful from each broth culture was inoculated onto blood agar, MacConkey agar (Oxiod), XLD agar (Oxiod) and Eosin methylene blue agar plates and incubated at 37ºC for 24 hours. Isolated colonies were identified morphologically, microscopically and biochemically according to **Holt *et al*., (1996)**.

For primary detection of *Pasteurella spp.* smears were taken from lungs, liver and heart blood at autopsy and were stained with Giemsa and Gram stains for detection of Gram negative bipolar coccobacilli of *Pasteurella.* For each pasteurella isolate, two mice were inoculated intra-peritoneally with 0.5 ml of overnight incubated broth culture of *Pasteurella species*. From dead mice, heart blood was smeared and stained with crystal violet for detection of *Pasteurella* bipolarity and inoculated on blood agar plates for re-isolation of *Pasteurella*.

Serotyping of *Salmonella* & *E. coli*

Salmonella isolates were serologically identified and serotyped according to Kauffman-White scheme (Kauffman, 1974) using polyvalent and monovalent O and H salmonella antisera. *E.coli* isolates were serotyped according to **Lennette, (1980)** using the available O antisera.

Antibiotic sensitivity tests

By using discs diffusion technique according to **Cruickshank *et al*. (1975)** and the interpretation according to NCCLS, 2002.

Pathogenicity tests of different isolated bacteria

A total of one hundred (100), one-day old chicks were used for testing the pathogenicity of different isolated bacteria. Chicks were divided into ten groups (10 chicks per group). Chicks in group 1 were kept as negative non-infected control, while chicks in groups 2,3,5,6,7,8, 9 and 10 were infected I/P with 0.1 ml of 24 hours broth culture per chick of the isolated *E.coli* (O2*,* the highest percentage of the isolates), *Protus mirabilis., Providenichia spp., Staphylococcus aureus, Salmonella typhimurium, Enterobacter cloacae, Aeromonas hydrophila* and *Shigella dysenteriae* respectively. Chicks in group 4 were reared for seventeen days then infected I/P with 0.1 ml of 24 hours broth culture per chick of *Pasteurella haemolytica* isolates. The chicks in all groups were fed on unmedicated ration and clean water and kept under observation for one week. Symptoms, mortalities and postmortem lesions were recorded. Re-isolation of the experimentally infected bacteria in each group was carried out.

Detection of *salmonella* in samples by PCR technique

Extraction of the DNA from the samples according EZ1 Virus Mini Kit v 2.0 (automated). The amplification reaction **(Oliveira *et al*., 2003)** was performed by using a DNA thermal cycler (T3 thermalcycler Biometra, Germany) where, all the reaction was carried out in a final volume of 25 µl in PCR tubes. The reaction mixtures consisted of 3µl of the extracted DNA template from the bacterial cultures plus 20 µl 1.1x PCR master mix (consisted of 1.25 units Taq DNA polymerase, 75 mM Tris-Hcl, 20mM (NH4)2So4, 1.5mM Mgcl2, 0.01%

(v/v) Tween® 20 and 0.2mM each of dATP, dCTP, dGTP and dTTP), add 1µl of forward and reverse primers (139 and 141). 40 µl of paraffin oil was added to avoid evaporation of the reaction mixture and the samples were subjected to preincubation at 94°C for 5 min., followed by 35 cycles of denaturation (94 for 1sec.), annealing (55°C for 1sec.), extension (72°C for 21sec.), and a final extension at 72°C for 7 min. The PCR products were stored in the thermal cycler at 4°C until they were used. 10µL of PCR product was analyzed by standard submarine gel electrophoresis (2% lonza agarose, 5v/cm) and 0.5µg/ml ethedium bromide **(Sambrook *et al*., 1989).** Visualization of the resulted product was done by location on gel documentation system (UV star 312nm, Biometra). One set of primers pairs was used. It was 139-141 specific for the invA genes (common gene for invasion of *salmonella*) which had specific sequence and conditions during PCR as shown in table (2 and 3). Molecular weight marker used named GelPilot 100 bp Ladder (100) cat no 239035 (Quiagen)

Table (2). Primers sequences used for detection of Salmonella species.

Primer	Target gene	Primer sequence (5´-3´)	G+c content	Length
139	*invA*	GTGAAATTATCGCCACGTTCGGGCAA	50%	26
141	*invA*	TCATCGCACCGTCAAAGGAACC	50%	22

Table (3). Primers conditions during PCR.

Primer	Forward primer sequence (5´-3´)	Reverse primer sequence (5´-3´)	Annealing temperature	Size of amplified product (bp)
invA	139	141	55c°	284

Detection of *Staph. aureus* in samples by PCR technique: according to **William *et al*., (2001)**. DNA to be amplified was released from whole cells by boiling. 1 ml of broth was centrifugated for 10 min at 16000 xg and the pellet was washed twice with sterile PBS, resuspended in 25 µL sterile PBS, incubated at 120°C for 15 min and then cooled on ice. After a final centrifugation for 2 min at 16000xg, supernatants were cooled at 4c° and used directly for PCR. Base sequences and predicated sizes of amplified products for the specific primers (from the midland certified reagent company, Inc.) used in this study are shown in Table (4). The amplification reaction was performed by using a DNA thermal cycler (T3 thermalcycler Biometra, Germany) and Quantitect prob RT-PCR kit. The reaction mixture consisted of 5µL of the extracted DNA template from the bacterial culture, 4.5 µL PCR grade water, 12.5 µL PCR master mix (containing hot start Taq DNA polymerase, Quantitect prob RT-PCR buffer (Tris.cl, Kcl, $(NH_4)_2SO_4$), dNTP mix (dATP, dCTP, dGTP and dUTP of ultrapure quality) and 8mMMgcl_2), add 1µL of each primer at 25 Pmol concentration. The samples were subjected to cycling parameters (i) 94°C for 3.0 min, (ii) 94C◦ for 1.5 min, (iii) 55C◦ for 1 min, (iv) 72C◦ for 1 min, (v) 36 cycles of steps 2 through 4 inclusive, and (vi) 72°C for 10 min. 10 µL of PCR product was analyzed by standard submarine gel electrophoresis (2% lonza agarose, 5v/cm) and 0.5 µg/ml ethedium bromide. Visualization of the resulted product was done by location on gel documentation system (UV star 312nm, Biometra).

Table (4). Primers sequences used for detection of Staphylococal species.

Target gene	Primer		Size of amplified product (bp)
	5′	3′	
Staph.*clfA*	G C A A A A T C C A G - CACAACAGGAAACGA	C T T - GATCTCCAGCCATA ATTGGTGG	638

Results and Discussion

Bacteriological examination in the present study revealed that many bacterial isolates were isolated from the examined samples in which mixed infections were common. The identification of the isolated bacterial agents revealed the isolation of *E.coli, Protus mirabilis., Pasteurella haemolytica, Providenicia spp., Staphylococcus aureus, Salmonella typhimurium, Enterobacter cloacae, Aeromonas hydrophila* and *Shigella dysenteriae* as in (Table 5). The most common clinical signs showed by diseased birds were inappetance, diarrhea, loss of weight gain, depression, respiratory manifestation, holding their head downward and abdominal enlargement. Similar clinical signs were described in ostrich chicks by **Simon and Thomas (1996)**, **Afifi (2003)** and **Aly and Moursi (2003)**. The postmortem examination of dead birds revealed congestion of subcutaneous blood vessels, of the internal organs, enteritis, pericarditis, airsacculitis, pneumonia, omphalitis and impaction of proventriculus. These lesions similar to those recorded by **Kamel *et al*., (2000)** and **Abdel-Razik (2007)**. From the isolated bacterial agents, *E.coli* represented the major bacterial isolates from ostrich samples (37.8%) which coincided greatly with **Nasef *et al*., (2003)** and **Ali and Ibrahium (2004)** who isolated *E.coli* from ostriches by 40.4% and 40.3%, respectively. Single infection with *E.coli* constituted 16.6% of the isolates, while, 83.3% of the *E.coli* isolation was mixed with other bacterial agents, mainly *Protus mirabilis* and followed by *Providenicia spp.* and *Salmonella typhimurium*, respectively. These results agreed greatly with those obtained by **Abdel-Razik (2007)** who isolated *E.coli* mixed with *Protus mirabilis, Enterobacter cloacae* and *Salmonella typhimurium* from ostriches. From the total isolates, *Protus mirabilis* isolates constituted 27.7% and were mainly isolated from cases suffering from diarrhea. The isolation of *Protus mirabilis* from ostriches was recorded by many investigators **(Afifi, 2003; Aly and Moursi, 2003; Moursi *et al*., 2003** and **Abdel-Razik, 2007)**. *Pasteurella haemolytica* isolates constituted 12.1% and were mainly isolated from lung abscesses. This result agreed with that obtained by **Nasef *et al*., (2003)** who isolated *Pasteurella haemolytica* from water samples, rations and lung abscesses of ostriches with percentage of 10.7%. Available literature recorded salmonellosis as one of the important disease condition of farmed ostriches causing a lethal enteric disease in chicks less than 6 months of age **(Huchzermyer, 2002)**. In our study, *salmonella spp*. was isolated at a percentage 4.2% and serotyped as *Salmonella typhimurium*. This seems to agree nearly with the results obtained by Ali and Ibrahium (2004) who registered an incidence of 1.5%.

Protus mirabilis, Providenicia spp., Enterobacter cloacae, Aeromonas hydrophila and *Shigella dysenteriae* were not isolated alone but isolated in mixed with *E.coli*, *Salmonella typhimurium*, or *Pasteurella haemolytica*. As shown in table (6)

Table (5). Incidence and types of bacterial agents isolated from examined ostrich samples.

Bacterial isolates	Single infection		Mixed infection		Total	
	No	%*	No	%*	No	%**
E. coli	15	16.6	75	83.3	90	37.8
Proteus mirabilis	0	0	66	100	66	27.7
Pasteurella haemolytica	10	34.4	19	65.5	29	12.1
Providenicia spp.	0	0	13	100	13	5.4
Staph. aureus	2	16.6	10	83.3	12	5.04
Salmonella typhimurium	7	70	3	30	10	4.2
Enterobacter cloacae	0	0	8	100	8	3.3
Aeromonas hydrophila	0	0	6	100	6	2.5
Shigella dysenteriae	0	0	4	100	4	1.6
Total	34	14.4**	204	85.5**	238	100

*Percentage according to total number of the isolated species
** Percentage according to total number of the isolates.

Table (6). Types of bacterial agents isolated in mixed infections from examined ostrich samples.

Bacterial isolates	No	%*
E. coli+ Proteus mirabilis	60	58.8
E. coli+ Providenicia spp.	12	11.7
Pasteurella haemolytica+ Enterobacter cloacae	8	7.8
Pasteurella haemolytica+ Aeromonas hydrophila	6	5.8
Pasteurella haemolytica+ Shigella dysenteriae	2	1.9
Pasteurella haemolytica+ Staph. aureus	2	1.9
Pasteurella haemolytica+ Providenicia spp.	1	0.9
Salmonella typhimurium+ E. coli	3	2.9
Staph. aureus+ Proteus mirabilis	6	5.8
Staph. aureus+ Shigella dysenteriae	2	1.9
Total	102	100

* Percentage according to total number of the isolates.

Serotyping of the isolated *E.coli* revealed that they belonged to O2 (32.2%), O78 (26.6%), O114 (16.6%) and O126 (13.3%) while 11.1% of the isolated *E.coli* were untyped (Table 7). The highest percentages of the isolates were belonging to O2 then O78 followed by O114 and finally O126. These results agreed with that of **Kamel *et al*., (2001), Nasef *et al*. (2003)** and **Abdel-Razik (2007)** who recorded that the most commonly implicated *E.coli* serotypes being responsible for colibacillosis in ostriches were O1, O2 and O78. Serogroup O2 was isolated from ostrich chicks suffering from diarrhea **(Kamel *et al*., 2001and Nasef *et al*., 2003).** Only one available literature **Abdel-Razik (2007)** could be found concerning the isolation of *E.coli* O114 from ostriches.

Table (7). Serotyping of *E.coli* strains isolated from Ostrich.

O serotype	No. of isolates	% of isolation
O2	29	32.2%*
O78	24	26.6%*
O114	15	16.6%*
O126	12	13.3%*
untypable	10	11.1%*
Total	90	100%

*Percentage according to total number of *E.coli* isolates.

The results of pathogenicity tests of the isolated bacterial agents in one-day old and seventeen days old chicks (Table 8) revealed that *Pasteurella haemolytica* was a highly pathogenic isolate to seventeen days old chicks. It caused 100% mortalities and associated with lesions of severe septicemia. The microorganism reisolated from all the internal organs of dead chicks. Similar results were obtained by **Nasef *et al*., (2003)**. These results disagreed with **Youseif *et al*., (2001)** who recorded that isolates of *Pasteurella haemolytica* which were obtained from naturally infected ostriches were non pathogenic for chickens. The pathogenicity testing of *E.coli* and *Aeromonas spp.* isolates showed 70% mortalities one-day old chicks with symptoms of severe diarrhea. The lesions revealed severe watery enteritis. These results were in agreement with that obtained by **Youseif (1995)** who found that *E.coli* strains isolated from local and imported one day old chicks were highly pathogenic to three days old chicks (100% mortalities) after subcutaneous route of inoculation. They also agreed in a great extent with that obtained by **Nasef *et al*., (2003)** who found that pathogenicity test of *E.coli* and *Aeromonas spp* in one-day old chicks caused 90% mortalities.

Table (8). Pathogenicity tests of bacterial agents isolated from Ostrich.

Group No.	Bacterial agent	No. of infected birds	Age of infection (days)	Rout of infection	Mortality rate	
					No.	%
1	Control negative	10	One-day old	I/P	0	0
2	*E. coli*	10		I/P	7	70
3	*Proteus mirabilis*.	10	One-day old	I/P	6	60
4	*Pasteurella haemolytica*	10	17 days old	I/P	10	100
5	*Providenicia* spp.	10	One-day old	I/P	0	0
6	*Staphy. aureus*	10	One-day old	I/P	1	10
7	*Salmonella typhi*	10	One-day old	I/P	3	30
8	*Enterobacter cloacae*	10	On-day old	I/P	0	0
9	*Aeromonas hydrophila*	10	On-day old	I/P	7	70
10	*Shigella dysenteria*	10	One-day old	I/P	0	0

I/P = intra-peritoneal

The isolates of Proteus spp. produced 60% mortalities in one-day old chicks. The same results obtained by **Nasef *et al*., (2003)**.

Salmonella typhimurium and *Staphylococcus aureus* isolates found to be pathogenic for chicks causing 30% and 10% mortalities, respectively with symptoms of pyrexia and severe diarrhea and the lesions showed severe enteritis and congestion of internal organs included liver, heart and kidneys. The microorganism was reisolated from all the internal organs of the dead chicks. These results were in agreement with those recorded by **Abdel-Razik, (2007)**.

Results of in-vitro sensitivity testing of the isolated bacterial strains (Table 9) revealed variation in drug sensitivity. Fluoroquinolone antibiotics (ciprofloxacin, enrofloxacin and danofloxacin) were found to be highly effective against most of tested bacterial strains followed by gentamycin, colitin sulphate and sulphamethoxazole – trimethoprim. Our results agreed with those obtained by many authors **(Aly and Moursi, 2003, Ali and Ibrahium, 2004** and **Abdel-Razik, 2007)**.

Table (9). Antibiotic sensitivity tests of different bacterial agents isolated from Ostrich.

Bacterial agent	Types of used antibiotics and the sensitivity of the isolates									
	AMP	AML	CT	CIP	Dfx	ENR	GM	OT	P	SXT
E. coli	S	R	R	R	R	R	S	R	R	S
Proteus mirabilis	R	R	R	HS	HS	HS	S	R	R	R
Pasteurella haemolytica	R	R	S	HS	HS	HS	S	R	R	S
Providenicia spp.	R	R	S	HS	HS	HS	HS	R	R	R
Staph.aureus	R	R	R	S	S	S	R	R	R	R
Salmo.typhi	R	R	S	HS	HS	HS	S	R	R	S
Enterobacter cloacae ct.	R	R	S	HS	HS	HS	S	R	R	S
Aeromonas hydrophila.	R	R	S	HS	HS	HS	HS	R	R	S
Shigella dysenteria	R	R	S	HS	HS	HS	HS	R	R	R

AMP= ampicillin, AML= amoxicillin, Dfx= Danofloxacin, CIP= ciprofloxacin, P= penicillin, CT= colistin sulphate, ENR= enrofloxacin, GM= gentamycin, OT= oxytetracycline and SXT= sulphamethoxazole – trimethoprim**.** R= Resistant, S= Sensitive and HS= Highly sensitive

In this study, we have used the PCR technique for detection of *Salmonella* species in the original samples broth, the results showed that the PCR technique was more sensitive and more accurate to detect more positive samples than in the convential microbiological technique (CMT) as shown in table (10) and in figures (1 -2), where by the CMT we detected 10 Salmonella isolates, also for Staphylococcus aureus we detected 12 isolates by the CMT but by the PCR technique we detected 16 isolates. These results in agreement with some authors as Schrank *et al*., (2001); Olivera *et al*., (2002); Fratamico (2003) and Ali (2007) who reported that an imported invA based PCR for the specific detection of *Salmonella typhimurium* and it gave the technique rapidity and specificity. The high sensitivity and specificity of the PCR method that made it an attractive alternative to culture, and widely used in clinical and research environments. PCR technique has been developed in the last few years too rapidly and specificity for detected microorganisms in feces and inner organs of infected animals.

Table (10). Comparison between the use of SMT and PCR for detection of some bacterial agents isolated from Ostrich

Bacterial agent	CMT*		PCR	
	No	%	No	%
Salmonella spp	10	4.2	14	5.9
Staphy spp	12	5.1	16	6.8

* CMT= standard microbiological technique

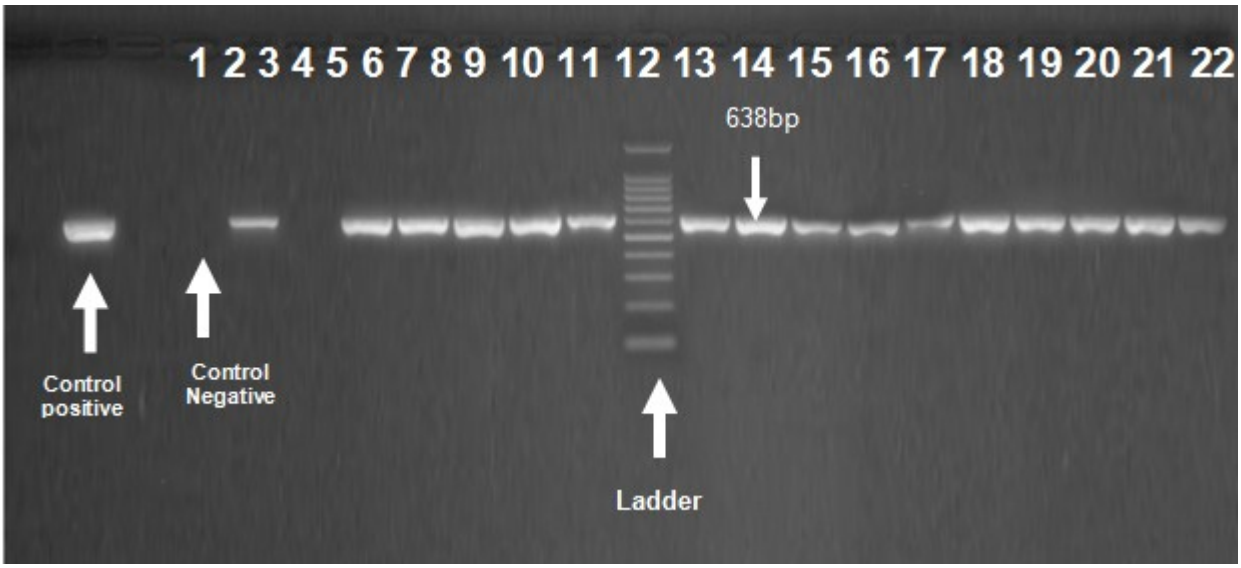

Fig. 1. Agarose gel electrophoresis showing positive amplification of 638 bp fragments using PCR was performed with primer specific for Staphy.*clfA*. Lane 2: Positive control (*staph aureus* NCTC 7447/ATCC® 6538P); Lane 4 Negative control (*E. coli* NCIMB 50034); Lane 12: 100 bp molecular weight ladder; Lanes 5, 7-11 and 13-22 show positive amplification of 638 bp fragment of *Staph* field isolates.

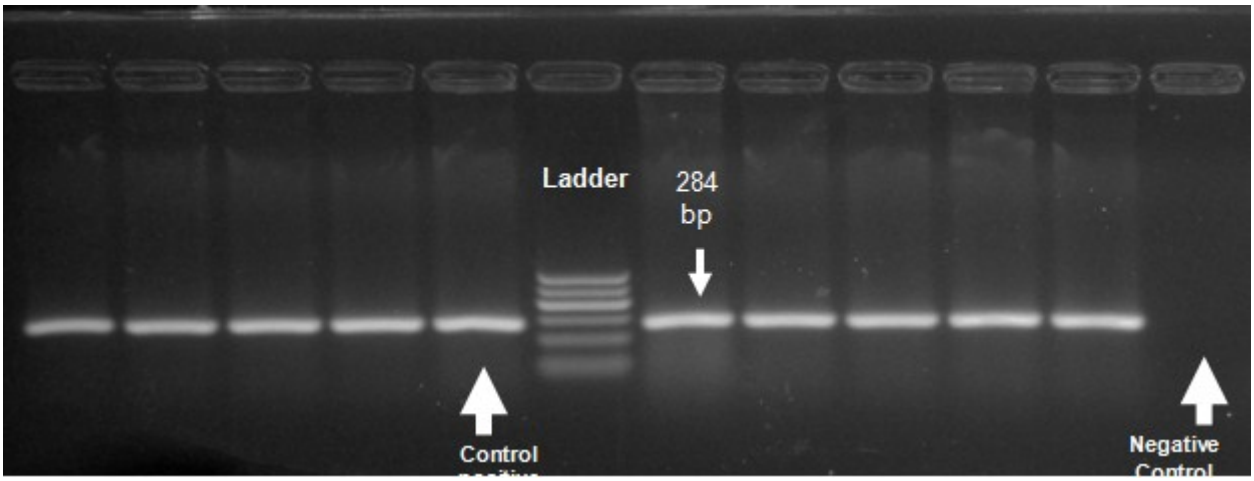

Fig. 2. Agarose gel electrophoresis showing positive amplification of 284 bp fragments using PCR was performed with primer specific for invA gene of *salmonella*. Lane 5: Positive control (*S.* Enteritidis ATCC 13076); Lane 12 Negative control (*E. coli* NCIMB 50034); Lane 6: 100 bp molecular weight ladder; Lanes 1-4 and 7-11 show positive amplification of 284 bp fragment of *salmonella* field isolates.

Finally, it may be concluded that the ostrich's farms should be subjected to the veterinary supervision for correct management, diagnosis and suitable medication. The diseased conditions in ostriches were usually caused by mixed bacterial agents. It is very important to improve hygiene and nutrition which probably acted as predisposing stress factors. Also, the early diagnosis of bacterial infections by the use of PCR technique for detection and the use of the effective antibiotics at the suitable dose are important to control the disease as early as possible.

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دراسات عن بعض البكتيريا الهوائية فى النعام فى مصر

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الملخص العربى

نظرا للتوسع فى انشاء مزارع النعام فى مصر ونظرا لانتاجية النعام العالية فى انتاج اللحوم والريش والبيض. بالاضافة لما تتميز به لحوم النعام من انخفاض نسبة الكولسترول بها. وايضا دهون النعام التى تستعمل فى صناعة بعض المراهم. لدلك لزم معرفة بعض المسببات المرضية البكتيرية. تم فحص ٢٠٠ عينة من النعام المريض والنافق حديثا من مزارع النعام المختلفة فى مصر وتم اجراء الصفة التشريحية وتجميع العينات اللازمة للعزل البكتيرى. اوضحت الفحوص البكتيريولوجية اصابة معظم الحالات المرضية باكثر من نوع من المسببات المرضية. وقد تم عزل كل من الميكروب القولونى، البروتيس، باستيريلا هيموليتكا، البروفدينشيا، المكورات العنقودية الدهبية، السالمونيلا تيفيميوريم، الانتيروباكتر، الاوروموناس والشيكلا من العينات التى تم فحصها. وقد تم تصنيف عترات الميكروب القولونى سيرولوجيا. وتم اجراء اختبار الضراوة للعترات المعزولة وتمت مناقشة الاعراض ونسب النفوق والصفة التشريحية بالتفصيل.وكذلك تم اجراء اختبار الحساسية للعترات المعزولة باستخدام المضادت الحيوية المختلفة ووجد انها حساسة للسبروفلوكساسين، الانروفلوكساسين، الدانوكساسين والجنتاميسين ثم السلفاميثوكسازول ترايميثوبريم. تم اجراء اختبار انزيم البلمرة المتسلسل على العينات ووجد انها اكثر حساسية من طرق العزل البكتيرية التقليدية.