

**DETECTION OF SALMONELLOSIS AND
PASTEURELLOSIS IN DUCKS USING POLYMERASE
CHAIN REACTION TECHNIQUE (PCR)**

*Ahmed M. Ammar**, *Salwa M. Helmy***, *Soad A. Abd El – wanis****
*and Nehal M. Nabil*****

* Bacteriology, Mycology and Immunology Dep., Fac. of Vet. Med., Zagzig Univ., Egypt.

** Bacteriology, Mycology and Immunology Dep., Fac. of Vet. Med.,
Kafrelsheikh Univ., Egypt.

*** Animal Health Research Institute, Dokki Lab., Giza, Egypt.

**** Animal Health Research Institute, Dakahlia branch, Egypt.

ABSTRACT

A total of 198 samples from internal organs and fecal matter of diseased and healthy ducks breeds were collected from farms and backyards in Dakahlia governorate (Egypt). The samples were examined bacteriologically and by PCR. Sensitivity, PCR and pathogenicity tests were applied. Fifteen samples (7.58%) were found to be positive for Salmonellosis and four strains were detected serologically (S. Infantis, S. Enteritidis, S. Inganda and S. Larochelle) also Untyped Salmonella strain was detected. Six samples (3.09 %) were found to be positive for Pasteurella multocida by bacteriological and PCR examinations.

INTRODUCTION

Heavy economic losses occur due to infection with Salmonellosis and it lead to morbidity, mortality, reduced egg and meat production in duck (*kumar and kaushi 1988*).

Salmonella is gram negative, nonspore-forming, usually motile, facultative anaerobic bacilli belonging to the family *Enterobacteriaceae*. Infection with *Salmonella* may or may not lead to a sometimes fatal Salmonellosis (*Ekperigin and Nagaraja 1998*).

Pasteurellosis is a serious bacterial disease that affects the duck industry and leads to severe economic losses. (*El-Sayed et al., 2000*).

Pasteurella multocida causes fowl cholera, a highly contagious and severe disease in water fowls. Cross-transmission of fowl cholera may happen between ducks and chickens, and vice versa. (*Mbuthia et al., 2008*).

MATERIAL AND METHODS

Samples collection:

A total of (198) samples were collected from farms and backyards in Dakahlia governorate (Egypt) as follow; 194 samples from duck backyards and 4 samples from duck farms). Samples for *Salmonella* isolation were from fecal matter and internal organs (liver, cecum and spleen) from healthy and diseased ducks while the lungs of these ducks were collected for the isolation of *Pasteurella* organism. The samples

were collected from different breeds such as khaki Campbell, Pekin, Muscovy, Mallard and Balady and Sudanese breeds. The samples were collected under aseptic condition on ice box and transferred to the laboratory.

Bacteriological examination:

1- Cultivation and isolation of *Salmonella* according to ISO 6579 (2002):

It was done by Pre-enrichment of the collected samples in Buffered Peptone Water as 1:10 dilution and then incubated aerobically at 37°C ±1°C for 18 hours ±2 hours. 0.1 ml was transferred to a tube containing 10 ml of the Rappaport Vassiliadis Soy broth and then incubated at 41.5°C ± 1°C for 24 hours ± 2 hours. One ml of the pre-enrichment culture were also transferred to a tube containing 10 ml of the Muller–Kauffmann tetrathionate/ novobiocin broth and then incubated at 37°C for 24 hours ± 2 hours. From the enrichment culture, 10 µl were inoculated onto the surface of Xylose Lysine Deoxycholate, Hektoen Enteric and MacConkey's agar plates then incubated at 37°C ± 1°C for 24 hours ± 2 hours. The plates containing characteristic colonies of *Salmonella* were selected and the gram staining test was performed. Each colony showing typical colonial appearance were subjected to biochemical identification and examined for hydrolysis of urea, H₂S production and Lysine decarboxylation.

Serological typing of *Salmonella* organism was performed according to (Kauffman, 1974) and (Cruickshank et al, 1975).

A- The isolates that were preliminarily identified biochemically as *Salmonella* were subjected to serological identification according to Kauffman-White Scheme.

B- Determination of somatic (O) and flagellar (H) antigens.

2- Cultivation and isolation of *Pasteurella multocida*:

For cultivation and isolation of *Pasteurella*, samples were suspended in Buffered Peptone water and then incubated at 37°C for 24 hours. From the pre-enrichment culture, 10 µl were inoculated onto the surface of blood agar and MacConkey's agar plates, and then incubated at 37°C for 24 hours. The plates containing characteristic colonies of *Pasteurella multocida* were selected and the gram stain test was performed. Each colony showing typical colonial appearance were subjected to biochemical identification and examined for hydrolysis of urea, H₂S production, Catalase, Indole, Oxidase production.

Antibiotic susceptibility testing according to (Finegold and Martin 1982).

Determination of the susceptibility of the isolated *Salmonella* and *Pasteurella multocida* organisms to antibiotic discs was adopted using the disc diffusion technique. The discs that used for *Salmonella* were oxytetracyclin, ciprofloxacin, enrofloxacin, ampicillin, amoxicillin, flumoquine, gentamycin, neomycin, chloramphenicol and doxycycline hydrochloride while the discs used for *Pasteurella multocida* were oxytetracyclin, ciprofloxacin, streptomycin, enrofloxacin, ampicillin, amoxicillin, erythromycin, gentamycin and penicillin.

Pathogenicity test:

Fifty SPF Pekin ducklings (one day old age) were floor reared and fed on a commercial ration suitable for their age and containing no antibiotics. They were divided into three groups, group 1 (20 ducklings) was infected at second day old orally with 10^3 - 10^{10} CFU of viable *Salmonella* organism (*Salmonella* Enteritidis) **according to Buchholz and Fairbrother (1992)** while group 2 (20 ducklings) was infected at 10 days old intranasal with 0.05 ml of viable *P. multocida* **according to Usha et al., (2010)** and group 3 (10 ducklings) was kept as a control. All ducklings were observed daily for 3 weeks after infection and subjected to clinical examination. Clinical signs and postmortem examination of dead ducklings were observed and reported daily. The internal organs (spleen, liver and cecum) for detection of *Salmonella* while (lungs and bone marrow) for detection of *Pasteurella multocida* in ducklings which died during the experiment and ducklings sacrificed at the end of the experiment were subjected to bacteriological examination.

Polymerase chain reaction technique:

DNA of the bacteria was extracted and specific primer for *Salmonella* organism was used according to (**Oliveira et al., 2003**).

Sequence of primer (forward GTGAAATTATCGCCACGTTCCGG GCAA)and(reverse TCATCGCACCGTCAAAGGAACC) while specific primer for *Pasteurella multocida* organism was used according to (**Deressa et al., 2010**), the sequence of primer (forward GCTGTAAAC GAACTCGCCAC) and (reverse ATCCGCTATTTACCCAGTGG).

DNA amplification:

DNA samples were amplified in a total of 25 µl as the following: 12.5µl of PCR master mix, 1µl of forward primer, 1µl of reverse primer, 5.5µl of PCR grade water and 5 µl of the template.

PCR cycling program:

For *Salmonella* organism according to (*Oliveira et al., 2003*), initial denaturation was at 94 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 1 second, annealing at 55 °C for 1 second and extension at 72°C for 21 second, with a final extension at 72 °C for 7 minutes while for *Pasteurella multocida* according to (*Deressa et al., 2010*), initial denaturation was 95°C for 3 minutes, followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 48°C for 1 minute, and extension at 72°C for 30 second).

Detection of PCR products:

Aliquots of amplified PCR products were mixed with gel loading buffer and electrophoresed in 1.5% agarose gel which prepared according to (*Sambrook et al., 1989*). The samples and a 100 bp DNA ladder (marker) were loaded in the wells in amount of 8µl of sample with 3µl of loading buffer and introduce 8 µl of the ladder. A current of 80 V for 1 hour was passed on the medi horizontal electrophoresis unit. Specific amplicons were observed under ultraviolet transillumination, compared with the marker. The gel was photographed by a gel documentation system.

RESULTS

Table (1): Incidence of isolated *Salmonellae* from fecal samples and internal organs collected from duck farms and backyards.

No. of tested samples		Cultural examination		Percentage of positive samples
		Negative	positive	
No. from backyards	194	180	14	7.58 %
No. from farms	4	3	1	
total	198	183	15	

Table (2): Incidence of isolated *Pasteurella multocida* from lungs collected from duck farms and backyards.

No. of tested samples		Cultural examination		Percentage of positive samples
		Negative	positive	
No. from backyards	194	188	6	3.09%
No. from farms	4	4	0	
total	198	192	6	

Table (3): Breeds of ducks examined for *Salmonella* and *Pasteurella multocida* organisms from duck farms and backyards.

Breeds of ducks	<i>Salmonella</i> isolation	
	No. of positive	Percentage of positive
Balady (Sudanese)	8	4.04%
Khaki Campbell	4	2.02%
Pekin	3	1.52 %
Muscovy	0	0
Mallard	0	0

Pasteurella multocida was isolated from Balady ducks only with a percentage of 3.09 %.

Table (4): Cultural, morphological and biochemical characters of the isolated *Salmonellae* and *Pasteurella multocida* according to (*Douglas Waltman et al., 1998*) and (*Richard et al., 1998*).

Organism Character	Cultural characters	morphological characters	biochemical characters
<i>Salmonella</i>	On XLD, <i>Salmonella</i> appeared as smooth colonies with black center while on Hektone enteric it appeared as Deep blue colonies but on MacConkey's agar appeared as Pale, colorless smooth, transparent, raised colonies.	Gram negative, non spore forming short rod shaped bacilli.	-Urea agar (negative - yellow color) and TSI agar (Positive - Gas, H ₂ S production, yellow butt (acidic) and red slant (alkaline). LI agar (positive) No gas, no H ₂ S production, deep purple (alkaline) slant and alkaline butt.
<i>Pasteurella multocida</i>	on blood agar appear as pin point non hemolytic with seminefrous odor but no growth appears on MacConkey's agar	gram negative, non spore forming rod shaped with bipolar staining	Urea agar (negative, yellow color), TSI agar (no H ₂ S), Indole test (positive, dark red ring), Catalase test positive (effervescence) and oxidase test positive, change of colony color.

Table (5): Results of serotyping of the isolated *Salmonella* from duck farms and backyards and breeds that have infection.

Type of the isolated strains		Number	Percentage of positive samples	Breed of ducks
Duck backyards	<i>S. Infantis</i>	2	13.3%	Pekin- Balady
	<i>S. Enteritidis</i>	3	20%	Balady
	<i>S. Inganda</i>	2	13.3%	Pekin- Balady
	<i>S. Larochele</i>	3	20%	Balady
	Untyped <i>Salmonella</i>	4	26.7%	Pekin- Khaki Campbell
Duck farms	<i>S. Inganda</i>	1	6.7%	Pekin

Table (6): Result of the sensitivity tests for the isolated *P. multocida*.

Antibiotic disc	OT	G	CF	ENR	AML	P	E	S	A
Susceptibility	R	S	S	S	R	R	R	S	R

R= resistant, S= sensitive, OT= Oxytetracyclin, G= Gentamycin, CF= Ciprofloxacin, ENR = Enrofloxacin, AML = Amoxicillin, a = Ampicillin, P = penicillin, E = Erythromycin, S = Streptomycin

Table (7): Results of the sensitivity tests for the isolated *Salmonellae*.

Antibiotic disc Strain	ENR	UB	G	OT	AML	C	CF	N	A	DO
	<i>S. Infantis</i>	S	S	S	S	S	S	S	S	S
<i>S. Inganda</i>	S	S	S	R	S	S	S	R	S	S
<i>S. Enteritidis</i>	S	S	S	R	S	S	S	S	S	S
<i>S. Larochele</i>	S	R	S	R	S	S	S	R	S	S
Un typed <i>Salmonella</i> (1)	S	S	S	R	S	S	S	R	S	S
Un typed <i>Salmonella</i> (2)	S	S	S	R	S	S	S	S	S	R
Un typed <i>Salmonella</i> (3)	S	S	S	R	R	S	S	R	R	S
Un typed <i>Salmonella</i> (4)	S	S	S	R	S	S	S	S	S	R

R= resistant, S= sensitive, ENR= Enrofloxacin, UB= Flumequine, G= Gentamycin, OT= Oxytetracyclin, AML= Amoxicillin, C= Chloramphenicol, CF= Ciprofloxacin, N= Neomycin, A= Ampicillin, DO= Doxycyclin hydrochloride

Table (8): Results of polymerase chain reaction of the isolated *Salmonellae* and *Pasteurella multocida*.

Organism	No. of positive samples	Amplification product (bp)
<i>Salmonella</i>	15	284
<i>Pasteurella multocida</i>	6	460

Results of Pathogenicity test:

Clinical signs:

After oral inoculation of *Salmonella* Enteritidis clinical signs appeared as loss of appetite, increase thirst, dullness, depression, diarrhea, in coordination of movement, inability to stand, death occurred within four days of symptoms and fecal excretion of *Salmonella* Enteritidis began after two days of oral inoculation.

After intranasal inoculation of *Pasteurella multocida* clinical signs were depression, light greenish diarrhea, dullness, respiratory manifestations (rales with nasal discharge from nostril) and deaths occur after 5 days of infection.

Post-mortem findings:

1- Post-mortem findings due to *Salmonella* Enteritidis.

Ducklings dying after the first week of infection developed typical picture of septicemia (congestion of all internal organs and petechial hemorrhages in liver and spleen) while those sacrificed at the end of the experiment showed enlarged gall bladder, enlarged kidney, some duckling showed congested liver while others show brownish liver, spleen was congested and the heart also congested.

Pasteurella multocida:

2- Post-mortem findings due to:

Ducklings dying during the experiment showed congestion in lung and other internal organs and pneumonia while those sacrificed at the end of the experiment showed septicemia (congestion at all blood vessels of internal organs), liver show hemorrhagic patches, petechial hemorrhages at the heart, air sacs were turbid, spleen was congested, kidney was congested, the content of the intestine was dark greenish, discharge at the upper respiratory tract and the lung was congested and had pneumonia.

Re isolation of *Salmonella* Enteritidis and *P. multocida* from experimentally infected ducklings:

All duckling that died during the experiment and ducklings that sacrificed at the end of the experiment were subjected to bacteriological examination and the two organisms were re isolated.

DISCUSSION

Results obtained in Table (1) revealed that 15 samples from duck farms and backyards were found to be positive to *Salmonella* isolation with percentage of (7.58 %), *Shamoon et al., (1998)* isolated *Salmonella* from digestive tract of ducks breed in open houses and it was 10 out of 60 birds (16.6%).

The results in Table (2) revealed that 6 samples from duck backyards were found positive to *Pasteurella multocida* with a percent (3.09%) while all farms were negative and this percent differ from *Xin (1995)* who examined 2500 ducklings and reported *P. multocida* with an incidence of 85%.

In Table (3) 15 positive samples for *Salmonella* organism (8 samples (4.04%) were from Balady (Sudanese) breed, 4 (2.02%) from Khaki Campbell duck breed and 3 (1.52%) from Pekin duck breed also one duck farm of Pekin breed was positive while 6 positive samples (3.09%) for *Pasteurella multocida* all of them from Balady (Sudanese) and in Table (5) *S. Infantis* and *S. Inganda* were reported in Pekin and Balady breeds but *S. Enteritidis* and *S. Larochelle* reported in Balady breed while untyped *Salmonellae* were reported in Pekin and Khaki Campbell breeds but **Rahman at al., (1999)** reported *S. Enteritidis* in Khaki Campbell and super M breed while **Simko (1988)** isolated *S. Enteritidis* with percentage of (1%) from duck farms but **Edes et al., (1994)** isolated *P. haemolytica* together with *Clostridium perfringens* (4 cases) and without *Clostridium perfringens* (6 cases) from the intestinal content of Muscovy breed.

From table (6) *Pasteurella multocida* was found to be sensitive to gentamycin, enrofloxacin, streptomycin and ciprofloxacin while it was resistant for oxytetracyclin, amoxicillin, penicillin, erythromycin and ampicillin while **Shivachandra et al., (2004)** reported that *P. multocida* was sensitive to chloramphenicol followed by enrofloxacin, lincomycin, norfloxacin and doxycycline-HCl and resistant to sulphadiazine.

In table (7) all strains were sensitive to ciprofloxacin, ampicillin, doxycyclin hydrochloride, chloramphenicol, amoxicillin, gentamycin and flumequine. The great variation in the sensitivity of the isolated *Salmonella* serotypes to different antibiotics support the report of **Viaene et al., (1970)**.

However *Salmonella* strains were sensitive to ampicillin and gentamycin which agreed with *Siddique et al., (1987)* who reported that *Salmonella* was sensitive for the same antibiotics and resistant for tetracycline, tylosin, trimethoprim and furazolidone.

Results in Table (8) revealed that *Salmonella* examination giving PCR product of 284 bp size and this agreed with *Siddique et al., (2009)* used same gene. Also in this table *P. multocida* giving PCR product of 460 bp size which agreed with *Deressa et al., (2010)*.

The postmortem examinations showed septicemia and congestion in the internal organs while *Yu et al., (2001)* infected two groups of ducklings artificially via subcutaneous inoculation of *Riemerella anatipestifer* and concluded that *Riemerella anatipestifer* infection is a septicemic disease but *Bang and Jun (2003)* made an experimental infection of *Pasteurella anatipestifer* and the pathological changes in infected ducks were brain hyperaemia and haemorrhage, serous pericarditis, fluffy heart, congestion of liver and spleen and liver necrosis.

Also in this study experimental infection of pekin duckling orally by 0.2 ml *S. Enteritidis* in second day old and clinical signs with post-mortem lesions and mortalities were reported and *Salmonella* was re isolated from the inoculated duck while *Buchholz and Faibrother (1992)* recorded that the inoculation of 10 day old mallard ducks orally and intravenously with *S. Pullorum* at selected concentrations 10^3 to 10^{10} colony-forming units give no signs or mortalities of the inoculated ducks and viable bacteria were cultured from livers of four mallards. Mallards undergo a short, sub clinical infection that was resolved without lasting

tissue damage. Concerning results of reisolation of *Salmonella* from experimentally infected duckling, *Salmonella* was re isolated from fecal matter and internal organs such as liver and cecum and spleen but *Gatellani et al., (1970)* reisolated *Salmonella* from the brain of experimentally infected ducks.

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تحديد الإصابة بالسالمونيلا والباستيريلا في البط باستخدام اختبار البلمرة المتسلسل (بى سى ار)

* أ.د/ احمد محمد احمد عمار ، ** أ.د/ سلوى محمود حلمى

*** د./ سعاد عبد العزيز عبد الوئيس ، **** ط.ب/ نهال محمود نبيل محمد

* قسم البكتريا والفطريات والمناعة- كلية الطب البيطري- جامعة الزقازيق - مصر

** قسم البكتريا والفطريات والمناعة- كلية الطب البيطري- جامعة كفر الشيخ - مصر

*** معهد بحوث صحة الحيوان- الدقى - مصر

**** معهد بحوث صحة الحيوان - فرع الدقهلية - مصر

نظرا لأهمية البط في صناعه الدواجن فقد يصاب البط بمعظم الأمراض التى تؤثر سلبيا على هذه الصناعة كما انه يلعب دور كبير فى نقل الأمراض المشتركة إلى الإنسان مثل التيفود فقد أجريت هذه الدراسة على سلالات مختلفة من البط لتحديد الإصابة بمرضى التيفود والكوليرا ومعرفة المضادات الحيوية التى من دورها أن تحد من هذه الأمراض و لذلك فقد تم تجميع ١٩٨ عينه من الأعضاء الداخلية مثل (الكبد، الطحال، الرئة والمعى الأعور) بالإضافة إلى زرق الطيور من المنازل والمزارع فى محافظه الدقهلية حيث جاءت النتائج كالأتي: نسبة الاصابة بمرض التيفود (السالمونيلا) ٥٨, ٧٪ و كانت هذه النسبة من البط البلدى والبكىنى والكمبل أما الباستيريلا مالتوسيدا فكانت بنسبه ٣٠٩٪ من سلالة البط البلدى للتربية المنزلية وجاءت المزارع سلبيه لها.

وتم تصنيف عترات السالمونيلا فقد تبين أنها سالمونيلا انفانتس, سالمونيلا انترتيدس, سالمونيلا

انجاندا, سالمونيلا لاروشيل و سالمونيلا غير مصنفة و لوحظ ان عترات السالمونيلا حساسه لبعض من

المضادات الحيوية مثل الجنتاميسين- الانروفلوكساسين - الكلورامفينيكول والسبروفلوكساسين بينما

Detection Of Salmonellosis And Pasteurellosis In Ducks Using ...

كانت بعض العترات مضاده للفلومكوين - الاوكسى تتراسيكلين - النيوميسين و الدوكسي سيكلين وكانت عتره واحده مضاده الاموكسيسيلين و الامبيسيلين اما بالنسبه للباستيريلا مالتوسيدا فكانت النتيجه انها حساسه للجنتاميسين و الانروفلوكساسين والاستربتومييسين و السبروفلوكساسين ولكنها كانت مضادة للاوكسى تتراسيكلين - الاموكسيسيلين - الامبيسيلين - البنسيلين والاريسروميسين. تم أيضا إجراء اختبار البى سى ار للتشخيص السريع والدقيق كما تم إجراء العدوى الاصطناعية فى البط لمعرفة الأعراض والصفة التشريحية.

تم مناقشة النتائج و الدور الذي تلعبه هذه الميكروبات فى صناعه الدواجن والتشخيص السريع لها لضمان عدم انتشار هذه الأمراض بين الطيور المختلفة وإنتاج سلالات خاليه من الأمراض.