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Dept. of Poultry Diseases,
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Head of Dept. Prof. Dr. S. Mousa.

MICROBIAL AGENTS INCREMINATED IN REDUCED HATCHABILITY OF DUCK EMBRYOS I. ISOLATION, IDENTIFICATION AND WHOLE CELL PROTEIN PROFILE

(With 3 Tables and 2 Figures)

By

R.S. IBRAHIM; ASHGAN M. SAYED*;
AZHAR M.ABDEL AZIZ* and A.M. SAYED*

* Animal Health Research Laboratory, Assiut.

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المسببات البكتيرية المسئوله عن انخفاض معدل الفقس فى أجنة بيض البط
١ -العزل والتعرف الميكروبى وتوصيف المحتوى البروتينى الخلوى السطحى

رجب سيد ابراهيم ، أشجان محمد سيد ، أزهار محمد عبدالعزيز
أشرف محمد سيد

عند استقصاء مشكلة انخفاض معدل الفقس فى مزرعة بط لانتاج بيض التفريخ عزلت عدة عوامل بكتيرية وكانت النسبة الكلية للبكتريا المعزولة ٥,٢% (سالمونيلا) ، ٦,٤% (الميكروب القولونى) ، ٦% (السيدوموناس) ، ٤,٤% (بروتياس) ، ٤% (كليبسيللا) ، ٤,٤% (ستروباكتر) ، ١٠% (انتيروباكتر) و ٢,٨% (الميكروب المكور العنقودى) من بيض البط الغير مخصب بالاضافة الى البيض الكابس. هذا و قد اعطت المحاولات المبذولة للعزل الفطرى نتائج سلبية. اظهرت هذه الدراسة والتى تم فيها عزل ميكروب السالمونيللا ايميك للمرة الأولى فى مصر تجانس جميع العترات فى المحتوى البروتينى السطحى والتى تدل على قوة الشبه بين العترات المعزولة وقد تميزت حلقات البروتين السائدة بالاوزان الجزئية ١١٦,٠٠٠ ، ٨٤,٠٠٠ ، ٧١,٠٠٠ ، ٥٥,٠٠٠ ، ٤٢,٠٠٠ ، ٣٦,٥٠٠ كيلو دالتون. بخصوص عترات الميكروب القولونىفقد اشتركت فى حلقات البروتين السطحى السائد الذى يحمل الاوزان الجزئية ٤٨,٥٠٠ ، ٤٨,٠٠٠ ، ٣٦,٥٠٠ و ٢٦,٦٠٠ كيلو دالتون.

SUMMARY

Decreased hatchability problem in duck breeder farm was investigated and several bacterial agents were isolated. The total percentages of isolated bacteria from infertile duck eggs and dead in-shell-embryos were (10%) *Enterobacter* spp., (6.4%) *E. coli*, (6%) *Pseudomonas* spp., (5.2%) *Salmonella* spp., (4.4%) *Proteus* spp., (4.4%) *Citrobacter* spp., (4%) *Klebsiella* spp., and (2.8%) *Staphylococcus* spp. Trials for mycotic isolation gave negative results. *Salmonella enteritidis serovar emek* which was isoalted for the first time in Egypt during this study, showed homogenous surface protein profile, indicating the strong homology of the isolated strains. The major protein bands were 116.000, 84.000, 71.000, 55.000, 42.000 and 36.500 Kda. *E. coli* isolates shared a common molecular weight surface protein profile of 48.500, 48.000, 36.500, 26.600 Kda.

Key Words: *Microbial, Agents, Reduced, Hatchability of Duck Embryos*

INTRODUCTION

Hatching of embryonated duck eggs influences by several factors, either environmental, management or due to infectious agents. Diseases transmitted via the eggs resulting in infertility or dead in-shell-embryos, are due to dirty egg-shell and/or egg-shell penetration by microbial agents. Many reports on isolation of different microbial agents were described. In Egypt El-Gharib et al., (1993) reported on isolation of *Enterobacteriacae, Staph.* as well as fungal agents. Shouman and Moustafa (1975) investigated the epidemiology of *Salmonella* infection in ducklings. This subject was studied world wide by Sarakbi (1983),Gajdsis (1985) and Liao and Xie (1988).

In the present study, the authors tried the isolation of bacterial and fungal agents from breeder duck eggs with high percentage of unhatched embryos in addition to throw some light on surface protein profiling with special reference to the most important isolated microbial agents.

MATERIALS and METHODS

Birds and sampling:

A breeder duck farm at Assiut Province suffered from high percentage of reduced hatchability. About 250 duck eggs did not hatch. All eggs were opened after surface disinfection with 2% tincture iodine. One hundred and ninety of examined eggs were dead in-shell embryos and sixty were infertile eggs. Microbial isolation was done from liver and yolk sac in case of dead in-shell embryos while from egg yolk in case of infertile eggs.

Bacteriological and mycological isolation:

Culturing was done on Nutrient and Selenite F broth (Oxoid), both cultures were incubated at 37°C for 24 hours, followed by subculturing on MacConkey's agar, S.S. agar (Oxoid), 10% blood agar and Nutrient agar (Oxoid). In the same time, a loopfull of embryonic fluids of all examined embryos were cultured on Sabouraud Maltose agar medium containing 0.5 mg/ml chloramphenicol, incubated at 25°C for five days for mycological isolation.

Identification:

Cellular morphology and motility test were done by Gram staining and semisolid agar stabbing, respectively. Gram negative bacteria were subjected for the following biochemical tests "indol prodution, urease test, triple sugar iron (TSI), H_2S production, citrate utilization, methyl red and Voges Proskauer (VP). Sugar fermentation pattern of glucose, lactose, sucrose and mannitol were done. Gram positive bacteria were tested for haemolysis on blood agar, growth on MacConkey's agar, coagulase test, urease test and pigment production.

Serotyping:

Serotyping was done for suspected *Salmonella* isolates on behalf of Centeral Health Laboratory, Ministry of Health, Cairo, Egypt on the basis of Kauffmann white scheme.

Sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE) of *Salmonella emek* and *E. coli* isolates:

Samples for SDS-PAGE were prepared from bacterial cell surface. Strains of *Salmonella emek* and *E. coli* which recovered in this study were cultured on Tryptone Soy Agar (TSA, Oxoid), incubated at 37°C for 18 hours.

A single colony of each isolate produced on TSA plate, was inoculated into 15 ml of Brain Heart Infusion (BHI) broth (Oxoid) and incubated at 37°C for 20 hours. The broth cultures were centrifuged at 12.000 rpm for 20 minutes. The bacterial cells were washed three times with PBS (PH 7.2) and suspended in 2 x sample buffer of [0.5 M tris. HCl (PH 6.8) containing 2-mercaptoethanol, 10% SDS, glycerol and 0.05% bromophenol blue]. The proportion of bacterial pellet and sample buffer were 1: 3 (weight per volume, w/v) and then placed in boiling water bath for 10 minutes, (Blackall et al., 1990).

SDS PAGE was performed by application of 10 µL of prepared samples per lane using Mini-Protean II electrophoresis cell (Bio-Rad Lab., Richmond, CA, USA) by the method of Laemlli (1970). The slab was prepared using 4% stacking gel and 10% separating gel which consisted of 1.5 M Tris-Hcl (PH 8.8), 10% SDS, acrylamide Bis (30%), 10% ammonium persulphate and TEMED. Running condition was at 30 V for 45 min. in stacking gel and 120 V for 80 min. in separating gel. The running buffer (PH 8.3) consisted of 1.5% Tris, 7.2% glycine and 0.5% SDS. The gel was stained for overnight with shaking by 0.1% coomassie brilliant blue R-250 (Pharmacia, LKB Bio technology, Uppsala, Seweden) in fixative solution (40% methanol and 10% acetic acid). Destaining was done using destaining solution until clear background was obtained. Approximate molecular weights were determined by comparing the mobility pattern of sample with that of prestained molecular weight standard marker (Sigma chemical Co.).

RESULTS

* Two hundred and fifty unhatched duck eggs were either infertile eggs (60) or dead in-shell embryos (190), revealed isolation of several organisms. Those organisms are gram negative bacteria (*Salmonella* spp., *E. coli* spp., *Pseudomonas spp.*, *Proteus* spp., *Klebsiella* spp., *Citrobacter* and *Enterobacter*) according to their biochemical and sugar fermentation features. Gram positive *Staph.* (*Staph. areus* and *Staph. epidermidis*) were also recovered and identified according to haemolysis,

coagulase test and pigment production. Results are illustrated in Tables 2 and 3.

* Isolation of fungi were negative in the present study.
* *Salmonella* isolates were belonged to *Salmonella enteritidis* group C_3 Kaufman White Schema and expressed as *ser emek* (somatic antigen 8, 20 and flagellar antigen phase I, phase II g.m.s.,-).
* Incidence of isolation of different bacterial spp. from both infertile eggs and dead in-shell embryos are shown in Table 1.

SDS-PAGE patterns of whole cell surface protein of *Salmonella emek* and *E. coli* are shown in Figs. 1 and 2. The electrophoretic patterns of *Salmonella emek* and *E. coli* isolates revealed homogenous protein profile. Regarding. *Salmonella emek*, there were minor protein bands at 33.000, 21.000, 18.000 and 16.000 Kda while major protein bands were demonstrated at molecular weight area of 116.000, 84.000, 71.000, 55.000, 42.000 and 36.600 Kda. In case of *E. coli* the major protein bands were observed at molecular weight area of 48.500, 48.000, 36.500 and 26.600 Kda, while minor bands were observed at 116.000, 78.000, 58.000, 41.000, 31.000, 24.000 and 22.000 Kda.

DISCUSSION

Trial for elecidating and clarifying the possible microbial etiologic agents of reduced hatchability in breeder duck farm was carrid out. The present study revealed the isolation of *Salmonella emek* (5%), *E. coli* spp. (6.7%), *Pseudomonas* spp. (10%), *Proteus* spp. (6.7%), *Klebsiella* spp. (3.3%), *Citrobacter* spp. (5%), *Enterobacter* spp. (10%), and *Staphylococcus* (3.3%) from infertile eggs. In case of dead in-shell embryos the same micro-organisms were isolated, *Salmonella emek* (5.3%), *E. coli* spp. (6.3%), *Pseudomonas* spp. (4.7%), *Proteus* spp. (3.7%), *Klebsiella* spp. (4.2%), *Citrobacter* spp. (4.2%), *Enterobacter* spp. (10%), and *Staph. areus* and *Staph. epidermidis* (2.6%). All *salmonella* isolates recovered from this study were *Salmonella emek*. By tracing the literatures back, it is worth to mention that this serotype was isolated for the first time in Egypt. No fungal isolation was obtained in this study.

Several authors in Egypt and allover the world reported on the microbial agents isolation from embryonic mortalities. In Egypt, Azzam (1998) concluded that aerobic bacterial agents associated with drop in fertility and hatchability with variable degree are *E. coli*, *Pseudomonas*, *Klebsiella*, *Proteus* and *Staph. spp.* with low incidence of *Salmonella* in contrast to our results. In other study El-Gharib (1991) isolated the same bacterial agents in addition to fungal agents. Isolation of *Proteus* was reported by Karaman (1980), Orajaka and Mohan (1985) and Safwat et al. (1986) from nonfertile eggs and dead in-shell embryos. Venkanagauda et al. (1996) in India could recover many isolates from early died chicks, those genera were *E. coli* (56.8%), *Proteus* (7.5%), *Salmonella* (6.8%), *Pseudomonas* (6%), *Klebsiella* (3.7%), *Enterobacter* and *Citrobacter* in addition to *Staph* (5.3%). These findings agree to great extent with our findings with some incidence variations.

Zagaevski (1956) explained the infection of eggs by egg-shell penetration after 6 - 7 days of incubation. While Liao and Xie (1988) recovered *E. coli* from reproductive tract of breeding ducks associated with decreased hatchability and low egg production. This is may explain that vertical transmission of *E. coli* is through genital tract of breeding ducks.

Regarding the isolation of *Salmonella spp.*, Zagaeveski (1956), Chowdhury et al. (1976) and Ali et al. (1987) recovered many *Salm. spp.* from infertile egg and dead embryos of ducks. So as far Egypt is concerned, Shouman and Moustafa (1975) could isolate different spp. of genus *Salmonella* including (*S. pullorum*, *S. gallinarum*, *S. typhi*, *S. meunschen*, *S. manhatten*, *S. anatum* and untyped *Salmonella spp.* of group C from dead in-shell duck embryos and one day-old-ducklings but they were unsuccessful to recover *S. emek*. Only one available literature on isolation of *Salmonella emek* was cited by Matsushita et al. (1996) where they recovered non-typhoidal Salmonellae in Tokyo from 1990 till 1994. One of these serovars was *S. emek* and described as imported serovar.

Several authors, El-Atereby (1982), Sarakbi (1983) and Sokkar et al. (1985) reported on isolation of *Klebsiella spp.* from unpipped duck embryos or from hatchability losses and conferming to our results.

Isolation of *Staph.* was reported by Dhawedkar and Dhanesar (1960), Sato et al. (1961), Karim and Ali (1980), Sokkar et al. (1985) and El-Gharib et al., (1993) from dead embryos and fresh eggs. In our present study we could not isolate any of fungal spp., while on opposite side Saif and Refai (1977), Sambyal et al. (1981), Sokkar et al. (1985) and El-Gharib et al., (1993) could recover *Aspergillus flavus*, *A. fumigatus* and *Penicillium spp.* from duck eggs and hatcheries.

SDS-PAGE profile of whole cell surface protein of *Salmonella emek* and *E. coli* revealed strong homology among all isolates in each species, suggesting that infection was caused by one serotype of both microbial agents. *Salmonella emek* isolates showed the same protein profile, where there are major protein bands at 116.000, 84.000, 71.000, 55.000, 42.000 and 36.500 and minor protein bands at 33.000, 21.000, 18.000 and 16.000 Kda molecular weights. In case of *E. coli* isolates, there were major protein bands at 48.000, 36.600 and 26.600 Kda and minor bands at 116.000, 78.000, 58.000, 41.000, 31.000, 24.000 and 22.000 Kda.

Delong and Manning (1994) described a fimbrial protein of *E. coli* with molecular weight of about 32.000 Kda with a great value in adhesion of highly pathogenic strains. The authors in the present study found the same molecular weight protein bands, but further work is needed to support the adhesion assay or relationship. In a comprehensive study Nico Overbeeke and Benlugtenberg (1980) reported that the outer membrane protein pattern of *E. coli* is characteristic in that usually from two to five major bands are observed in the molecular weight range between 20.000 and 42.000 Kda. Similar results also were observed in the present study but additional work are required to find the relationship between serotypes and outer membrane protein profile.

Regarding to *Salmonella spp.* Poppe et al. (1993) support our results where they characterized 318 *S. enteritidis* strains that were isolated mainly from poultry depending on outer membrane protein (OMP) and lipo-poly-saccharides (LPS) profiles. They concluded on relationship of these properties to one another, and their diagnostic and pathogenic significance. They found that 35 of 36 strains possessed the same OMP profile.

In brief, many records on microbial aetiology were tried including isolation, identification, but more recent criteria are required for strict identification such as plasmid profile, lipopolysaccharide profiles (LPSP), outer membrane protein profile (OMPP), DNA homology. Such features may differentiate the isolates that belong to the same serotype, indicating the subserotypes variations and may explain the unusual behaviour as well as virulence factors of microbial infections.

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Table1. Frequency of bacterial isolates from both infertile eggs and dead in-shell embryos.

Type	Total	%	Infertile eggs		Dead in-shell	
	Rate		Rate	%	Rate	%
Salmonella	13/250	5.2	3/60	5	10/190	5.3
E. coli	16/250	6.4	4/60	6.7	12/190	6.3
Pseudomonas	15/250	6	6/60	10	9/190	4.7
Proteus	11/250	4.4	4/60	6.7	7/190	3.7
Klebsiella	10/250	4	2/60	3.3	8/190	4.2
Citrobacter	11/250	4.4	3/60	5	8/190	4.2
Enterobacter	25/250	10	6/60	10	19/190	10
Staphylococcus	7/250	2.8	2/60	3.3	5/190	2.6
Total	**108/250**	**43.2**	**30/60**	**50**	**78/190**	**41**

Table2. Differentiation of isolated *Staph.* spp.

Test	*Staph. Aureus* (N = 3)	*Staph. Epidermidis* (N = 4)
Haemolysis	B-haemolysis	-ve
MacConkey's growth	-ve	-ve
Coagulase	-ve	+ve
Pigment production	+ve	-ve

Table 3. Biochemical tests and sugar fermentation reactions of different isolated bacterial species.

Test	*E. coli*	*Salmonella*	*Klebsiella*	*Pseudomonas*	*Proteus*	*Citrobacter*	*Enterobacter*
Gram stain	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Motility	+	+	+	+	+	+	+
MacConky's agar	+	+	+	+	+	+	+
V.P.	-	-	+	-	-	ND	ND
Indol	+	-	-	ND	V	+	-
TSI	Y/Y	Y/R	Y/Y	ND	Y/R	Y/R	Y/Y
H_2S	-	+	-	-	+	-	-
Citrate	-	+	+	+	+	+	+
MR	+	+	-	-	+	+	-
Glucose	+	+	V	-	+	V	ND
Lactose	+	+	+	-	-	V	ND
Sucrose	V	-	+	+	+	V	ND
Mannitol	+	+	+	-	-	+	ND

V: Variable reactions
ND: Not determined
Y/Y: Yellow butt versus yellow slant
Y/R: Yellow butt versus red slant.

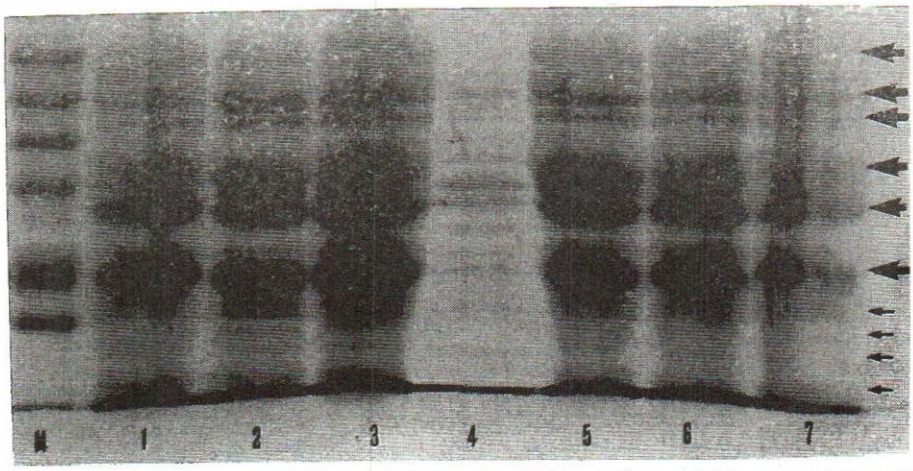

Fig. 1. SDS-PAGE profile of whole cell surface protein of *Salmonella enteritidis ser. emek* (Lane No. 1-7). M: Prestained molecular weight marker. Thick arrow indicates the major protein bands of 116.000, 84.000, 71.000, 55.000, 42.000 and 36.600 Kda molecular weights. Thin arrow arrow indicates the minor protein bands of 33.000, 21.000, 18.000 and 16.000 Kda molecular weights from top to bottom.

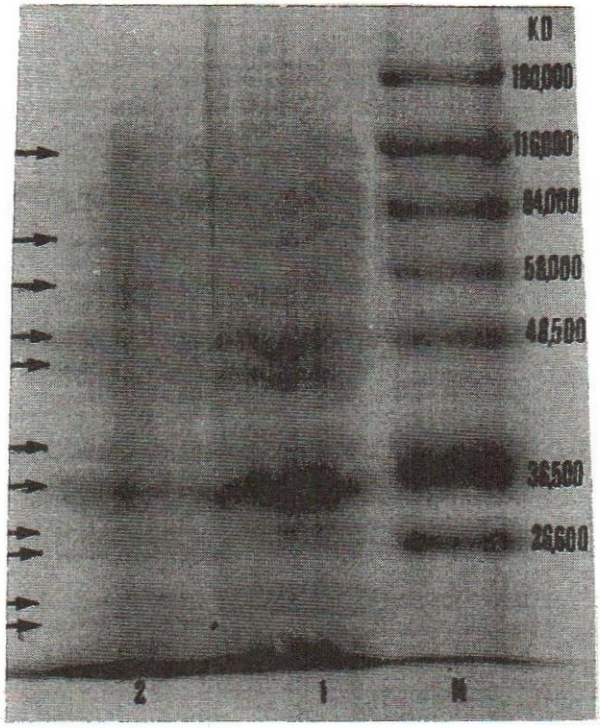

Fig. 2. SDS-PAGE profile of whole cell surface protein of *E. coli* isolates (Lane No. 1 and 2). M : prestained molecular weight marker. Arrows represents the protein bands molecular weights of 116.000, 78.600, 58.000, 48.500, 48.000, 41.000, 36.500, 31.000, 26.600, 24.000 and 22.000 Kda from top to bottom.