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THE IMPACT OF TEMPERATURES TO REDUCE THE RISK OF SALMONELLA ARIZONA AND SALMONELLA ENTERITIDIS IN TABLE EGGS

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ABSTRACT

The prevention of salmonellosis is closely associated with food safety. So, in the present study A survey was conducted to determine the prevalence of *Salmonella* in 300 hen's eggs (commercial and balady) representing 150 eggs for each were collected randomly from Assiut city. Every 5 eggs represent one sample. From balady egg's shell 6.67%, 13.33% S. arizona and 3.33%, 10% *S. enteritidis* were recovered using S.S. (salmonella shigella) and XLD (Xylose Lysine Desoxycholate) agars, respectively. *Salmonella arizona* was detected in balady egg's content in percentage 0, 6.67% while, *S. enteritidis* detected in 3.33 and 6.67% on the same media. Commercial farm hen eggs came secondary to Balady hen eggs. *S. arizona* and *S. enteritidis* could be detected on egg shell at a same percentage 3.33% on S.S. agar but on were XLD they isolated by 6.67, 10%, respectively. From egg content both microorganisms detected in 3.33% on both media but failed to detect *S. enteritidis* on S.S. agar. Serologically other Salmonellae detected were *S. typhimurium, S.anatum* and *S. kentucky. S. arizona* and *S. enteritidis* subjected in this study to antibiotic sensitivity test. Antibiotic resistance in relation to 9 antibiotics (Doxveto (Dov, 30µg), Lincomycin (L2, 2mcg), Novobiocin (NV30, 30mg), Neomycin (Neo, 30mcg), Amoxyveto (VMD, 5mcg), Eryton (CIN, 15mcg), Ciprofloxacin (Cip, 5mcg), Cloxacillin (CX1, 1mcg) and Cephradine (CE30, 5mcg) was studied. The results indicated that *S. arizona* and *S. enteritidis* were sensitive to Doxveto (Dov, 30µg) and Novobiocin (NV30, 30mg) and resist to the remained antibiotics. This work was conducted to study the effect of different degree of temperatures on *S. arizona* and *S. enteritidis* in hen's eggs. The results indicated that *S. arizona* still found until the fifth week in the inoculated eggs with test organisms stored at 4°C, while *S. enteritidis* still found until the third week. While, after immersing the inoculated eggs with test organisms in boiling water bath for 10 and 15 minutes then cooled and examined the results indicated that complete destruction of *S. arizona* and *S. enteritidis*. It could be concluded that we must keep eggs refrigerated at all times and Eggs should be cooked at least ten minutes. The economic and public health importance of *S. arizona* and *S. enteritidis* that affect the human health through consumption of eggs were discussed. Likewise, suggestive measures for improving the quality of produced eggs and the suitable procedure to cook eggs are given.

Key words: *Table eggs, Salmonella arizona, S. enteritidis.*

INTRODUCTION

Despite of the extensive public health measures over the past century, *Salmonella* remains the second most commonly identified cause of bacterial foodborne disease in the developed countries and a signficant cause of morbidity and mortality in the developing world (WHO, 2002 and Amin, 2004). In Egypt salmonellae were found in 3% cases of children diarrhea in rural areas and 4% in urban areas. In Upper Egypt, salmonellae were detected in 14.8% of cases of children diarrhea (FAO, 1993)

Table eggs which constitute several dishes or foods consumed and are considered cheap sources of protein, have served as vehicles for numerous enteropathogens (Adesiyun *et al.,* 2007). *Salmonella* spp., particularly *S. arizona* and *S. enteritidis* have been most frequently associated with table eggs (Nygard *et al.,* 2004). Table eggs contaminated by bacterial pathogens and consumed raw or improperly cooked have been responsible for many epidemics of gastroenteritis worldwide (Nunes *et al.,* 2003). *S. arizona* is known to cause infection in reptiles and other animals. It is an uncommon human pathogen. Over the last 50 years, approximately 50 case reports and case series have been appeared (Cone *et al.,*

1990). *S. enteritidis* continued to be a major cause of illness and death. It is the most common serovar causing approximately 80% of foodborne salmonellosis cases (CDC, 2009). Moreover, it results in more deaths than any other pathogen (Olsen and Hammack, 2000).

A variety of foods and environmental sources harbor bacteria that are resistant to one or more antimicrobial drugs used in human or veterinary medicine and in food-animal production (Anderson *et al.,* 2003 and Schroeder *et al.,* 2004). Though many bacteria recovered from poultry or poultry-related samples have been monitored, few published studies have reported on antimicrobial resistance in bacteria, particularly *Salmonella* recovered from shell eggs (Chung *et al.,* 2004 and Dias de Oliveira *et al.,* 2005). So in our study try to monitor the antibiotic susceptibility of isolates.

Moreover, *Salmonella* penetration can occur either after lay (most likely) or at later stages in the distribution chain, if eggs are subject to environmental changes resulting in temperature differential across the shell, or condensation. Certain combinations of temperature and RH can lead to condensation on the eggshell, and influence survival, penetration and growth of *Salmonella* (Radkowski, 2002). Messens *et al.* (2007) provided that the cold chain is maintained, commencing cooling at farm level has the highest beneficial effect with regard to the control of the growth of *Salmonella.* Also, survival behaviors of heat shocked cells of these pathogens by applying different temperatures were studied by several investigators (Bradshaw *et al.,* 1990 and Korashy *et al.,* 2008).

So, the present work aimed to isolate *S. arizona* and *S. enteritidis* from hen's eggs (commercial and balady), study the antibiotic susceptibility of isolates and to control the infection through study the effect of refrigerator temperature on isolates for storage and different temperatures during hen's egg preparation for consumption.

MATERIALS and METHODS

A- Collection of samples:
300 of fresh hen eggs of native breeds (Balady) and poultry farms (commercial) (150 for each) bought from different groceries in Assiut city. Every 5 eggs constitute one group.

B- Preparation of samples:
Egg shells were tested by rinse for shell surfaces as described by Moats (1980). Egg contents were prepared and evacuated according to Speck (1984).

C- Isolation of *Salmonella* spp. from egg samples
Samples were pre-enriched on Rappaport-Vassiliadis (RV) broth at 37ºC for 24 hours (Wallace *et al.,* 2009). S.S. agar and XLD agars were used to isolate Salmonella spp. according to Andrews and Hammack (2001) and Wallace *et al.* (2009). Pink colonies with black center on S.S. agar as well as yellow colonies with or without black centers colonies on XLD agar were identified as Salmonella spp. by Gram stain and various biochemical tests as described by Andrews and Hammack (2001) and ISO-6579: 2002 standard. Modification of the confirmatory process indicated by FDA (1995, 2002) protocols was done.

D-Serological identification of Salmenollae species:
Isolates proved biochemically to be *Salmonella spp.* were subjected to serological identification according to Kauffmann white scheme (Kauffmann, 1974) by using rapid diagnostic *Salmonella* antiserum sets. Isolates were sub-cultured on nutrient slope for 24 hours at 37°C for application of slide agglutination technique, two homogenous suspensions were made on a slide by suspending a piece of suspected colony in a drop of sterile physiological saline. A drop of each of separate O and H Salmonella factors were added separately to each of the suspensions with standard loop thoroughly mixed to bring the microorganisms in close contact with antisera. Positive agglutination occurred within a minute and could be easily seen with the naked eye. A delayed or partial agglutination was considered as negative or false result.

Determination of O (somatic) antigens:
Separate O antisera were applied to determine the group of the Salmonella isolates.

Determination of H (flagella) antigens:
Polyvalent H antisera for both phase I and phase 2 were tried in order to determine the complete antigenic formula of the isolates.

E- Antibiotic susceptibility test
S. arizona and *S. enteritidis* isolates were tested for antibiotic resistance using the standard disc diffusion method (NCCLS, 1993). Discs containing Doxveto (Dov, 30µg), Lincomycin (L2, 2mcg), Novobiocin (NV30, 30mg), Neomycin (Neo, 30mcg), Amoxyveto (VMD, 5mcg), Eryton (CIN, 15mcg), Ciprofloxacin (Cip, 5mcg), Cloxacillin (CX1, 1mcg) and Cephradine (CE30, 5mcg) were used. The multiple antibiotic resistance (MAR) index for each isolate was determined, it was defined as a/b, where a is the number of antibiotics to which a particular isolate is resistant and b is the number of antibiotics to which the isolates is exposed (Adesiyun *et al.,* 2007).

D- Effect of different temperatures on *S. arizona* and *S. enteritidis* inoculated in eggs:-

(Chantarapanont *et al.,* 2000; Korashy *et al.,* 2008 and EFSA, 2009):

The tested strains were purified and inoculated into brain heart infusion broth and overnight incubated. For 0.1 ml of the incubated broth, sterile saline was added to bring turbidity to 0.5 McFerland Standard. Pervious suspension is the standard strain suspension of which 1 ml may contain approximately about $1x10^5$ CFU (Quinn *et al.*, 1994).

Preparation of an egg for inoculation by the test organisms:

A small hole was made at the blunt end of an egg with a sterile drill. Aseptically injection of an inoculum (1 ml) containing 1×10^5 c.f.u of the tested organism into the egg yolk by a sterile needle. Then the hole is covered after injection by Ducocement.

1- **Cooling**: The inoculated eggs with test organisms were stored at $4^{\circ}C$ until deterioration of eggs which detected by testes of freshness of egg (candle lamp and dipping in water bath).

2- **Boiling**: The inoculated eggs with test organisms were immersed in boiling water bath for 10 and 15 minutes then cooled and examined.

RESULTS

The results were illustrated in the following Tables

Table 1: Incidence of *Salmonella* spp. in the examined Balady hen's eggs samples:

Samples	No. of examined samples	Media used	Presumptive *Salmonella* colonies		Positive *Salmonella* spp. serologically		*S. arizona*		*S. enteritidis*	
			No.	%	No.	%	No.	%	No.	%
Egg shell	30	S.S.agar	10	33.3	5	16.67	2	6.67	1	3.33
		XLD agar	14	46.67	8	26.67	4	13.33	3	10
Egg content	30	S.S.agar	8	26.67	3	10	0	0	1	3.33
		XLD agar	12	40	6	20	2	6.67	2	6.67

Table 2: Incidence of Salmonella spp. in the examined commercial hen's eggs samples:

Samples	No. of examined samples	Media used	Presumptive Salmonella colonies		Positive Salmonella spp. serology		*S. arizona*		*S. enteritidis*	
			No.	%	No.	%	No.	%	No.	%
Egg shell	30	S.S.agar	8	26.67	5	16.67	1	3.33	1	3.33
		XLD agar	11	36.67	8	26.67	2	6.67	3	10
Egg content	30	S.S.agar	9	30	4	20	1	3.33	0	0
		XLD agar	13	43.33	8	26.67	1	3.33	1	3.33

Table 3: Frequency distribution of different isolated Salmonella strains in the positive hen's egg samples based on their serological identification.

Isolated Salmonella strains	Balady eggs				Commercial eggs			
	Egg shell		Egg content		Egg shell		Egg content	
	SS	XLD	SS	XLD	SS	XLD	SS	XLD
Salmonella arizona	2	4	0	2	1	2	1	1
Salmonella enteritidis	1	3	1	2	1	3	0	1
Salmonella typhimurium	1	0	2	2	0	1	0	2
Salmonella anatum	1	0	0	0	2	2	2	1
Salmonella kentucky	0	1	0	0	1	0	1	3
Total	5	8	3	6	5	8	4	8

Table 4: Antibiotic sensitivity tests for S. arizona and S. enteritidis

Drug used	MAR index	Drug sensitivity pattern	
Amoxyveto VMD	Non		Resist
Cephradine CE30	Non		Resist
Ciprofloxacin Cip	Non		Resist
Cloxacillin Cx1	Non		Resist
Doxveto Dov	2.3	sensitive	
Eryton Cin	Non		Resist
Lincomycin L2	Non	Resist	
Neomycin Neo	Non		Resist
Novobiocin NV30	2.3	sensitive	

Table 5: Results of effect of different temperatures on *S. arizona* and *S. enteritidis* inoculated in eggs

Temperature	***Duration***	***Survival of organisms***	
		S. arizona	***S. enteritidis***
Cooling	*1st day*	+	+
	3rd day	+	+
	1 week	+	+
	2nd week	+	+
	3rd week	+	+
	4th week	+	-
	5th week	+	-
Boiling	*10 min.*	-	-
	15 min.	-	-

DISCUSSION

Salmonellosis is a foodborne infection of major economic importance. Contamination of table eggs with Salmonella, especially S. arizona and S. enteritidis, is a major health concern worldwide (Lublin and Sela, 2008). The present study showed that presumptive salmonella colonies using SS and XLD agar were 33.3, 46.67% from egg shell and 26.67, 40% from egg content of balady hen's egg, respectively (Table,1). While, from farm hen's egg shell presumptive salmonella colonies were 26.67, 36.67% and from content were 30, 43.33% on both media, respectively (Table 1,2). Salmonella spp. were previously isolated from egg samples in percentage 22.9% by Jean *et al.* (1995); Adesiyun *et al.* (2007) and 26.1% by Adesiyun *et al.* (2005) and Indar *et al.* (1998) from shell and content of table eggs. This may be attributed to the chickens carry the Salmonella in their own bodies, and pass Salmonella along to the yolk and white while the egg is forming in the ovaries. Chickens can also pass bacteria to the eggshell—and through the shell pores into the inner egg—when the egg is laid. Chickens can harbor Salmonella without being sick themselves. Salmonellae are frequently isolated from various farm environments such as water, feed, and manure. Moreover, asymptomatic shedding of Salmonella in feces of hens also occur so there is a risk of the pathogen reaching to eggs through fecal contamination (Troutt *et al.,* 2001 and Huston *et al.,* 2002). According to the European Commission (2003), eggs and products containing raw eggs are among the food categories most likely to pose the greatest risk to public health in relation to salmonellosis. In 2007, the reported number of cases and incidence of human salmonellosis in the EU were, respectively, 154,099 cases and 31.1 cases per 100,000 inhabitants (EFSA, 2009). Eggs and egg products were the most frequently reported source of foodborne outbreaks caused by Salmonella in 2006 (EFSA, 2007). S. enteritidis is the serovar causing more than 60% of the human Salmonella infections in the EU (EFSA, 2009), and also most often associated with egg borne infections (WHO, 2001).

The incidence of infection with *S. enteritidis* via hen eggs has increased in many countries (Yukiko *et al.,* 2001) and the risk of *S. arizona* increased through consumption of table egg. On the basis of biochemical and serological methods for identification of the isolates we found that *S. arizona* could be detected in balady hen's egg shell in percentage 6.67, 13.33% and from egg content 0, 6.67% by SS and XLD agar, respectively. and 3.33, 6.67% from egg shell, 3.33, 3.33% from egg content of commercial hen's egg (Table1,2). The low number of confirmed positive isolates compared with the total number of suspected colonies indicates that the identification of Salmonella should not based solely upon the morphological characters of suspected colonies on differential media (Abdel-hameid, 2013). Siebeling (1975), D'Aoust *et al.* (1990) and Sechter (1996) could detect *S.arizona* infection from fresh eggs.

In the United States *S. enteritidis* is the second most commonly isolated serotype from human illness, and is known to be strongly associated with shell eggs and egg containing products. Eggs can become contaminated internally either by penetration through the shell or directly during formation in the reproductive tract (Zoe *et al.,* 2012). In our study, *S. enteritidis* could be detected in egg shell of both balady and commercial hen's egg in the same percentage 3.33, 10% and from content of 3.33,6.67% of balady and 0,3.33% of commercial hen's egg by S.S. and XLD agar, respectively (Tables 1,2). *S. enteritidis* has been isolated from the yolk, albumen, and shell of naturally infected intact eggs by S.S. agar (Jean *et al.,* 1995). Yukiko *et al.* (2001) detected S. entertidis in 12/15 samples of naturally contaminated liquid egg and in 37/39 samples shell egg using XLD agar. Higher percentage of *S. enteritidis* could be isolated by Gantois *et al.* (2008); Lucía *et al.* (2012) (25%).

According to information gathered from 84 countries responding to a global survey conducted by the World Health Organization (WHO), *S. enteritidis* and *S. typhimurium* accounted for 70% of all human and nonhuman isolates of *salmonella* reported worldwide between 1995 and 2008 (CDC, 2009). More than 2,500 *Salmonella* serovars have been identified according to the serospecificities of the somatic and flagellar antigens. Some serovars, exemplified by *Salmonella enterica* serovar Typhimurium and *S. enteritidis,*can infect a broad range of hosts (Geimba *et al.,* 2004). Corresponding Table 3, it is persisted that the different identified strains of *salmonella* via sero-typing technique other than *S. arizona* and *S. enteritidis* were *S. typhimurium, S. anatum* and *S. Kentucky* in different percentages. Although, there are relatively low numbers of positive samples in this study, the pathogen represent a potential risk to consumers on the basis that all salmonellae are potentially pathogenic (Zansky *et al.,* 2002).

A prevalence of 22.9% for resistance to antimicrobial agents detected amongst Salmonella isolates from eggs is considerably lower than the prevalence of 50% reported for Salmonella isolates recovered from livestock in Trinidad (Adesiyun *et al.,* 1993). So, the antimicrobial resistance testing is highly discriminatory and might also give helpful information towards an effective therapy. Antibiotics used in this study represented the major groups of antibiotics used. The result of this test showed that both *S. arizona* and *S. enteritidis* were sensitive to

Doxveto (Dov, 30µg) and Novobiocin (NV30, 30mg) and resist to the remained antibiotics (Table 4). Similarly, the prevalence of resistance, as determined by resistance to one or more antimicrobial agents detected by Bajaj *et al.* (2003); NARMS (2005) and Musgrove *et al.* (2006), although higher than reported for isolates from table eggs by Brown *et al.* (1994) is significantly lower than found in Brazil (Simango and Mbewe, 2000). It has been reported that inappropriate use of antimicrobial agents in livestock may result in the development of resistance amongst bacteria in these animals or their products (Washington, 1979; Waltner-Toews and McEwen, 1994).

MAR index values of >0.2 are considered indicators of high-risk source of selective pressure for the development of antibiotic resistance bacteria (Krumperman, 1983). So, it is necessary to consider the resistance pattern of the *Salmonella* in question before administering any antibiotic.

As seen, high level of multi drug resistance of the *S. arizona* and *S. enteritidis* strains isolated from both types of egg samples is of concern to public health so, maintenance of proper cleanliness and hygiene during handling to limit the degree of contamination is essential together.

Eggs are one among the major animals foods mostly marketed raw and frequently consumed raw, semi-raw in many dishes and form an important part of meals contain raw eggs as an essential ingredient (homemade ice cream, mayonnaise, eggnog etc.). These dishes are not heated up to the (FAO, 1979) recommended temperatures, 155 ^{o}F for at least 15 seconds (Mermelstein, 2001) and this is not enough to render an egg free from pathogenic organisms as yolk is high nutritive medium permits multiplication of the organisms. Several methods of microbial destruction were discussed by Serrano *et al.* (1997) and Brackett *et al.* (2001). Table 5 clarified that cooling storage not effective on *S. arizona* and *S. enteritidis* if contaminate egg from beginning also, that in similar with (Baker, 1990; Radkowski, 2002; Messens *et al.,* 2006).

Braun and Fehlhaber (1995) observed that *Salmonella* can be found in the egg yolk within 1 or 2 days at 20°C and 30°C but also within 14 days at 7°C. But, boiling procedure used for destruction of the inoculated test organisms is enough at 10 and15 minutes. The results came in line with Baker *et al.* (1983); Schuman *et al.* (1997); Soliman and El-Tabiy (2007) and (Korashy *et al.,* 2008).

The obtained results recommended that boiling must be adopted for more than 10 minutes to ensure complete destruction of pathogens may contaminate eggs. Heat treatment – time temperature conditions aims to achive a decrease in the number of viable organisms (Stadelman *et al.,* 1996 and Schuman *et al.,* 1997).

There is a considerable demand for using high temperatures during cooking of eggs to destroy the present pathogens. Gossett and Baker (1981) studied the textural problems and greenish discoloration which affected eggs due to high temperatures used and suggested the addition of citric acid which gives favorable effects due to thermal destruction of microorganisms.

So, in order to remove or reduce the risk of some of pathogenic organisms of public health importance contaminate eggs, there are several points must be adopted. Chosen of healthy mother's hens are necessary to obtain eggs of free pathogens. Hygienic measures applied in the farms during handling and storage. Using of hot soapy water with those come in contact with eggs and egg containing foods in work areas. Eggs must be held at low temperature (5 ^{o}C) to prevent proliferation of the pathogens. Cleaning with sanitizer minimizes the contamination of the shells, beside pasteurization of egg products as statutory requirements in many countries. Educational programs for consumers informed the risks resulted from eating under cooked eggs particularly the elderly and immune–compromised persons who are more susceptible to infection.

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تأثير درجات الحرارة المختلفة للحد من خطورة السالمونيلا اريزونا والسالمونيلا انتريتيدز فى البيض التجارى

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تعتبر السالمونيلا من اخطر الميكروبات المسببة للأمراض التي تنتقل عن طريق الغذاء في العالم الثالث خاصة السالمونيلا المعوية حيث أنها تعتبر من المسببات الأساسية لحدوث المرض والوفاة وان نسبة الوفيات بها تكون أعلى من غيرها من الميكروبات ووجد أنها تمثل ٧٪ من الوبائيات التي حدثت عن طريق الغذاء. لذلك، في هذه الدراسة تم تجميع ٣٠٠ عينة من بيض الدجاج (المزارع والبلدى) يمثلون ١٥٠ بيضة لكل نوع جمعت عشوائيا من مدينة أسيوط. كل ٥ بيضات تمثل عينة واحدة. وتم عزل وتصنيف السالمونيلا سيرولوجيا فكانت نسبة السالمونيلا اريزونا ٦.٦٧٪ و١٣.٣٣٪من القشرة الخارجية و٦.٦٧,٠٪ من محتوى البيض البلدى بينما السالمونيلا انتريتيدز بنسبة و٣.٣٣٪، و ١٠٪، ٣.٣٣و٦.٦٧٪ من قشرة ومحتوى نفس العينات باستخدام S.S. and XLD agar على التوالى. اما عن عينات البيض التجارى فكانت النسبة اقل فقد تم عزل السالمونيلا اريزونا من القشرة بنسبة ٦.٦٧,٣.٣٣٪ والسالمونيلا انتريتيدز بنسبة ٣.٣٣، ١٠٪. بينما من محتوى العينات ٣.٣٣٪ لكل منهم على S.S. and XLD agar. كما تم إجراء اختبار الحساسية للعترات المعزولة باستخدام ٩ أنواع من المضادات الحيوية وقد أظهرت العترات مقاومة عالية لبعض المضادات الحيوية (مفردة أو مجمعة) من بينها Cephradine, Cloxacillin, Amoxyveto, Eryton, Ciprofloxacin , Neomycin وLincomycin فى حين أعتبر Novobiocin ، Doxveto هما الأكثر حساسية للعترات. الوقاية من السالمونيلا يرتبط بشكل وثيق مع سلامة الأغذية. لذا هدفت الدراسة الى معرفة تأثير اختلاف درجات الحرارة للحد من خطورة السالمونيلا اريزونا والانتريتيدز فى البيض على صحة الانسان. وقد أوضحت النتائج أن السالمونيلا أريزونا لا تزال موجودة حتى الأسبوع الخامس في البيض المحفوظ عند درجة حرارة ٤ درجة مئوية، في حين السالمونيلا انتريتيدز لا تزال موجودة حتى الأسبوع الثالث وهذا اثناء تخزينة. لكن عند سلق البيض قي حمام مائى عند درجة حرارة ١٠٠ درجة مئوية لمدة ١٠ و ١٥ دقيقة فقد تم القضاء على كل من السالمونيلا أريزونا وانتريتيدز. لذا يصنح بسلق البيض جيدا للقضاء على السالمونيلا وتجنب خطورتها.