Identity of *Salmonella* Enterica Serovar Enteritidis, Typhimurium and Kentucky Local Strains Used In Preparation of Polyvalent Inactivated Salmonella Vaccine in Chickens in Egypt.

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Abstract

Salmonellosis is one of the most important bacterial diseases affecting poultry. Its importance is derived from the loss in productivity in affected birds and the hazard it causes for public health. For preparation of polyvalent inactivated *Salmonella* vaccine, we tested the local virulent *Salmonella Typhimurium*, *Salmonella Enteritidis* and *Salmonella Kentucky* strains phenotypically by the microscope, biochemically by API test and genotypically by Polymerase Chain Reaction (PCR) using a specific primer for *fli*C gene of *Salmonella Typhimurium* and a specific primer for *sef*A gene of *Salmonella Enteritidis* and a specific primer for *inv*A gene of *Salmonella Kentucky*. In Conclusion: *Salmonellae* are gram negative rods. They are aerobic or facultative anaerobic organisms that ferment sugars, producing gas and H2S and that have a complex antigenic structure (somatic "O", flagellar "H", and capsular "K" antigens). Multiplex PCR reveals that *Salmonella Kentucky, Salmonella Typhimurium and Salmonella Enteritidis* were positive for *invA* gene with a product of 284bp, *fliC* gene with a product of 620bp and *sefA* gene with a product of 488bp respectively.

Introduction

Salmonellae are facultative intracellular pathogens that cause localized or systemic infections, in addition to their emphasis in chronic asymptomatic carrier state. They are of worldwide economic and public health significance as food born pathogen ***(Little et al., 2007)***. *Salmonellae* are ubiquitous, host-adapted or zoonotic pathogens ***(Smith, 2003)***.

These bacteria are rods from the *Enterobacteriace* family, and most of them are mobile. They are aerobic or facultative anaerobic organisms that ferment sugars, producing gas and H2S, and that have a complex antigenic structure (somatic "O", flagellar "H", and capsular "K" antigens) ***(Barrow, 2000).***

Both *Salmonella Enteritidis* and *Salmonella Typhimurium* are the

most important serotypes for salmonellosis transmitted from animals to humans yet, ***(Foley and Lynne, 2008).*** While poultry is the main animal reservoir of *Salmonella Kentucky* ***(Molla B, et al., 2006).***

-The aim of the present work is: identification of the locally isolated *Salmonella* strains which used in the polyvalent inactivated *Salmonella* vaccine preparation phenotypically and genotypically.

Materials and methods

Strains used:

Salmonella Typhimurium, Salmonella Enteritidis and *Salmonella Kentucky* local virulent strains were obtained from the reference strain bank at the Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Abassia, Cairo, Egypt.

Confirmation of *Salmonella* species strains:

A.* Cultivation on Salmonella Shigella agar (SS agar) *(Collier et al., 1998):

A Loopful from the selected culture was inoculated into tryptose broth then incubated at 37ºC for 24 hours. The inoculated broth was streaked onto the surface of Salmonella Shigella agar (SS agar) and incubated at 37ºC for 24 hours.

B. Morphological examination *(Crukshank et al., 1975):*

A separate colony was picked up and stained by Gram´s stain **(Cruickshank *et al.*, 1975)** then examined microscopically for detection of Gram negative bacteria which appear pink rods in its shape.

C. Biochemical Identification *(Murray et al., 2003):*

Pure culture of each strain was identified biochemically by using **API 20E** identification system (Biomerieux –France cat# 20-100) following the procedures of kit manual.

D. **Molecular identification of *Salmonella* species by PCR:**

1-* DNA extraction *(Sambrook et al., 1989; Oliveira et al., 2003):

According to the instruction of the used DNA extraction kit {Isolate Genomic DNA mini kit (Bioline, Cat. No. BIO-52032)}.

2- DNA Amplification *(Soumet et al., 1999):*

Detection of *invA* gene which is specific for *Salmonella Kentucky*, *fliC* gene which is specific for *Salmonella Typhimurium* and *sefA* gene which is specific for *Salmonella Enteritidis* by multiplex PCR.

a) Extraction of DNA:

Following the instructions of the used DNA extraction kit {Isolate Genomi DNA mini kit (Bioline, Cat. No. BIO-52032)}.

b) Running of PCR:

The PCR mix and cyclic conditions applied as described by ***Read et al. (1994)***. In a 0.5 ml PCR tube the following reaction mixture was prepared as 4 ul DNA template, 25 ul master mix, 1 ul forward primer (25 pmol), 1 ul reverse primer 25 pmol, and 19 ul double distilled water. The 50 µl reaction mixture

was placed in the thermal cycler and was programmed as 94 C° for 3 minas initial denaturation followed by 35 cycles 94 C° denaturation, 56C° annealing and 72C° extension then 72 C° for 10 min as final extension. The PCR products were stored in the thermal cycler at 4 C° until they were collected. The amplicons and 100 bp DNA ladder were run on 1% Agarose gel.

Table (1): *List of primers used in the multiplex PCR-based assay for the detection of Salmonella species* ***(Soumet et al., 1999):***

Target gene	Primer sequence (5′ ⟶ 3′)	Amplicon (bp)	Used for
***iro*B F** ***iro*B R**	TGCGTATTCTGTTTGTCGGTCC TACGTTCCCACCATTCTTCCC	606	Universal primer for detection of *Salmonella* species
***inv*A F** ***inv*A R**	GTGAAATTATCGCCACGTTCGGGCAATCATC GCACCGTCAAAGGAACC	284	Specific primer for detection of *Salmonella Kentucky*
***fli*C F** ***fli*C R**	CGGTGTTGCCCAGGTTGGTAAT ACTGGTAAAGATGGCT	620	Specific primer for detection of *Salmonella Typhimurium*
***sef*A F** ***sef*A R**	GATACTGCTGAACGTAGAAGG GCGTAAATCAGCATCTGCAGTAGC	488	Specific primer for detection of *Salmonella Enteritidis*

Results

Colonial Morphology:

Figure (1), demonstrates the characteristic colorless, smooth and round colonies with black center (due to H2S production) of the selected strains of *Salmonella* (*Enteritidis*, *Typhimurium* and *Kentucky*) on Salmonella Shigella agar medium after 24hr at 37°c.

Biochemical Confirmation:

Table 2 shows that, depending on the results of API 20E identification system, the three *Salmonella* strains used in the vaccine preparation had the same biochemical profile. The only difference was seen in the inositol fermentation test where *Salmonella Typhimurium* and *Salmonella Kentucky* were inositol positive as shown in **Figure (3)** while, *Salmonella Enteritidis* was inositol negative as shown in **Figure (2)**.

Molecular Confirmation:

The results showed that the genomic DNA of local *Salmonella* strains (*Enteritidis*, *Typhimurium* and *Kentucky*) was positive for *iroB* gene with a product of 606bp which specific for *Salmonella* species as shown in **Figure (4).** By multiplex PCR *Salmonella Kentucky, Salmonella Typhimurium and Salmonella Enteritidis* were positive for *invA* gene with a product of 284bp, *fliC* gene with a product of 620bp and *sefA* gene with a product of 488bp respectively as shown in **Figure (5).**

Fig (1) Salmonella enterica serover typhimuruim, Enteritidis and Kentucky on SS agar medium.

Table (4): *Biochemical confirmation of salmonella strains by API 20E system:*

Tests / Strain	ADH	LDC	ODC	CIT	H_2S	GLU	MAN	SOR	RHA	MEL	ARA	ONPG	URE	TDA	IND	VP	GEL	SAC	AMY	INO
S. Enteritidis	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
S. kentucky	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+
S. typhimurium	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+

Figure (2): Biochemical reactions of *S. enteritidis* in API 20E system.

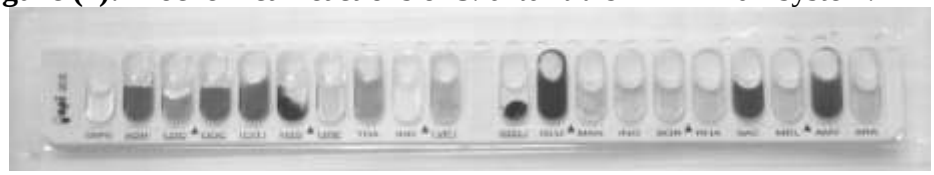

Figure (3): Biochemical reactions of *S. Kentucky* and *S. Typhimurium* in API 20E system

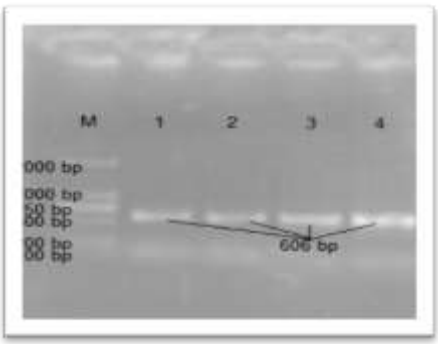

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Figure (4): Agarose gel electrophoresis showing amplification of 606 bp fragments of *Salmonella* species M: DNA marker; lane 1 positive control, lane 2 S enteritidis, lane 3 S. typhimurium and lane 4 S. Kentucky.

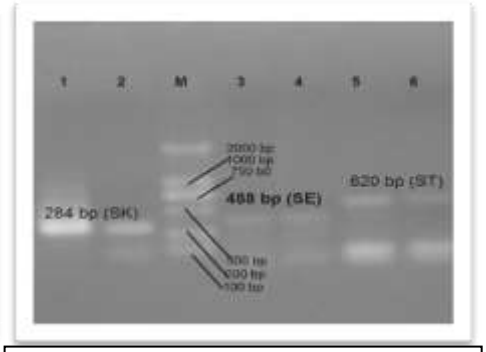

Figure (5): Multiplex PCR showing amplification of 284bp fragments of *S. Kentucky* in lane 1and 2 while lanes 3and 4 showing amplification of 488bp fragments of *S. Enteritidis.* Lane 5and 6 showing amplification of 620bp fragments of *S. Typhimurium*; M: DNA marker

Discussion

Poultry represents the most important source of cheap protein throughout the world. *Salmonellae* are responsible for considerable losses in the poultry industry production particularly in countries applying intense production systems ***(O'brien, 1988; and Lumsden, J.S., and B.N. Wilkie, 1992).***

Salmonella species are facultative intracellular pathogens causing localized or systemic infections, in addition to chronic asymptomatic carrier state. Although more than 2000 *Salmonella* serovars have been identified worldwide, only about a dozen serovars accounting for more than 65% of the isolates reported from human beings and poultry ***(Nagreja et al., 1991).***

Vaccination as part of a *Salmonella* control program contributes to the achievement of *Salmonella* free poultry meat and eggs. Mass poultry vaccination programs introduced to combat *Salmonella* infections have led to a dramatic fall in the number of cases since the late 1990s ***(APA, 2013)***. Vaccination appears to be the most specific control measure. For this reason considerable efforts have been made in the present work to develop polyvalent *Salmonella* vaccine, which would induce protective immunity in chickens and reduce the public health hazards.

According to the ***Egyptian standards for evaluation of veterinary biologics – CLEVB (2009)*** before vaccine preparation, complete morphological and biochemical identification followed by molecular characterization are required. The selected strains of *Salmonella Enteritidis*, *Salmonella Typhimurium* and *Salmonella Kentucky* showed characteristic colorless colonies with black center (due to H2S production) by culturing on Salmonella Shigella agar medium after 24 hours incubation at 37C. The biochemical identification using API 20E identification system revealed that, the three *Salmonella* strains which used in the vaccine preparation were all of *Salmonella Typhimurium, Salmonella Enteritidis* and *Salmonella Kentucky* had the same biochemical profile, the only difference was seen in the inositol fermentation test where *Salmonella Typhimurium and Salmonella Kentucky* were inositol positive but *Salmonella Enteritidis* was inositol negative. The same results were confirmed by ***(Mohamed, Amal, A. and Aly, Seham, M. 2008)*** who completely identified locally isolated salmonellae from poultry farms. Also ***Fatma Gad (2011)*** studied the phenotypic and genotypic characterization of the locally isolated salmonellae and concluded to the same finding. The same results were confirmed by ***(Mahmoud et al., 2010)*** who completely identified locally isolated salmonellae from poultry farms. Also ***(Shell WS et al., 2017)***

studied the phenotypic and genotypic characterization of the locally isolated salmonellae and concluded to the same finding.

As regards to PCR amplification, all Salmonella used strains were positive for amplification with gene specific for Salmonella species (iroB gene) with a product of 606 bp fragments as shown in **Fig. (4)**. The same results were achieved by ***(Sareyyupoglu et al, 2008)*** who used a specific primers for the *iro*B gene and obtained 606 bp fragments with all tested salmonella species including *Salmonella Typhimurium, Salmonella Enteritidis* and *Salmonella Kentucky*. Regarding the species specific primers, Multiplex PCR results showed in **Fig. (5)** revealed that *Salmonella Enteritidis* specific *sefA* gene primers was amplified at the molecular length of 488 bp while *Salmonella Typhimurium* specific *fliC* gene primers was amplified at the molecular length of 620-bp. The same results were achieved by ***(Sareyyupoglu et al, 2008)*** who used a specific primers for the (*iro*B) gene and obtained 606bp fragments with all tested *Salmonella* species including *Salmonella Typhimurium, Salmonella Enteritidis* and *Salmonella Kentucky.* And by multiplex PCR *S. Kentucky, Salmonella Typhimurium* and *Salmonella Enteritidis* were positive for *invA* gene with a product of 284bp, *fliC* gene with a product of 620bp and *sefA* gene with a product of 488bp respectively as shown in **Fig. (5).** At the same issue, ***(Mona I. El-Enbaawy et al., 2013)*** reported the same findings with the same organisms. ***(Zahraei et al, 2005)*** subjected a strains of *Salmonella* group C2 including *Salmonella Kentucky* to the specific (*inv*A) gene PCR and were confirmed as *Salmonella* positive by the predicted product a 284bp DNA fragment.

On conclusion, from the above mentioned data, the locally isolated and identified phenotypically and genotypically salmonella strains could be used for the preparation of polyvalent local inactivated vaccine for protection of chickens against field infection with *Salmonella Typhimurium, Salmonella Enteritidis* and *Salmonella Kentucky* which may achieve better efficacy than those either produced from the standard strains or commercially available from imported source.

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الملخص العربى

تعريف عترات السالمونيلا المستخدمة فى تحضير لقاح مثبط مركب للوقاية ضد السالمونيلا انتريتيدس و السالمونيلا تيفيميوريم و السالمونيلا كنتاكى فى الدجاج

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- السالمونيلا من اهم الميكروبات المسببة لمشاكل مرضية فى قطاع الدواجن و الذى يؤدى الى خسائر اقتصادية تتمثل فى حالات الوفيات وحالات الاصابات وتكلفة العلاج و الاعدامات فى مجازر الدواجن.وكذلك تاثيرها على الصحة العامة للانسان.

-وقد تم فى هذه الدراسة اختبار هوية عترات السالمونيلا عن طريق اجراء بعض الاختبارات المورفولوجية و البيوكيميائية وتم التاكيد عليها باجراء اختبار تفاعل البلمرة المتسلسل عن طريق تعيين نوع من الجينات المميزة للسالمونيلا وهو gene (*iroB*).

- وقد تم ايضا عمل تفاعل البلمرة المتسلسل المتعدد للسالمونيلا تيفميرم وكذلك السالمونيلا اانتريديس والسالمونيلا كنتاكى وتعيين الجينات المميزة لهم: gene (*sef*A) and gene (*inv*A) (*fli*C) gene) على التوالى. و بناء على هذه النتائج يوجد امكانية لإستخدام تلك العترات لتصنيع لقاح مركب مثبط محلى للحماية ضد ميكروبات السالمونيلا انتريتيدس و السالمونيلا تايفيميوريم و السالمونيلا كنتاكى فى الدجاج.