

**ISOLATION OF *SALMONELLA* SPECIES FROM BUFFALO CALVES MEAT AND PRELIMINARY EVALUATION OF ITS IRRADIATED VACCINE**

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**ABSTRACT**

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The bacteriological examination of 200 meat samples obtained from diarrheic buffalo calves revealed that *Salmonella* isolates could be recovered with an incidence 2 %. Serological identification of *Salmonella* species by using O and H antisera revealed that the most common *Salmonella* serovars were *Salmonella typhimurium* followed by *Salmonella dublin* and *Salmonella anatum* with an incidence 1 %, 0.5% and 0.5 % respectively. *Salmonella typhimurium* is inactivated by 3 KGy dose of irradiation of meat and also the isolated organism for preparation of the vaccine. The result of vaccination inoculation challenge studies revealed that the prepared *Salmonella typhimurium* vaccine was effective in protecting rabbits from *salmonellosis*.

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**Key words:** Buffalo meat irradiation, *Salmonella typhimurium* vaccine.

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**INTRODUCTION**

*Salmonella* is reported to be the most frequently diagnosed infectious cause of acute diarrhea in buffalo calves. Mortality from acute *salmonellosis* in calves may be as high as 60 %; so it is necessary to introduce urgent measures for control of the spread of *Salmonella* in calves (Gerdien *et al.*, 2007).

Bovine *salmonellosis* is a worldwide bacterial disease causes great economic and public health problems. *Salmonellosis* is a zoonotic disease causing severe invasive infection in human and it causes economic and welfare losses in infected herds; as the disease causes fever and the meat of the affected animal will not be fit for human consumption (El- Safey, 2013). *Salmonella* is a group of bacteria that can cause a diarrhoeal illness in humans. Most infected persons develop diarrhoea, fever, and abdominal cramps 6 to 72 h after infection. The illness usually lasts 4 to 7 days. However, too many people continue to get sick and die from eating contaminated food. The disease in calves usually occur between two to six weeks of age, although animals may become infected after birth, or with *S.dublin* may be born infected.

In Egypt, *Salmonella typhimurium* was recovered with an incidence ranging from 8.4%- 19.0% from calves. (Galal *et al.*, 2008). Protection of animals from *Salmonella* should have a top priority, so *Salmonella* reduction plan which depends on rapid

diagnosis and vaccination must be established (Nagy *et al.*, 2001).

Ionizing radiation is able to inactivate food borne pathogens on a variety of food products and can serve as a final critical control point to ensure the microbiological safety of foods (Kundu *et al.*, 2014).

Vaccines made with bacteria killed by gamma radiation, rather than by standard methods of heat or chemical inactivation, may be more effective, also may not need to be kept cold till the time of use; an advantage in setting where refrigerated vaccines are impractical or impossible (Raz *et al.*, 2006). Irradiation destroys the DNA, making the bacteria unable to replicate so it cannot establish an infection, but some residual metabolic activity may survive, so the irradiated bacteria can still find its natural target in the host. (Chaander *et al.*, 2007).

**Aim of the work:** Isolation and identification of *Salmonella typhimurium* from the meat of diarrheic buffalo calves and preparation of vaccine from it by using gamma radiation.

**MATERIALS and METHODS**

**Samples:** A total of 200 meat samples obtained from diarrheic dead buffalo calves, were examined for the presence of *Salmonella typhimurium* at El-Dakahlia Governorate, the samples were sent with a minimum delay to the laboratory (Department of Radiation

Health Researches) for investigation under aseptic precautions and sent to the lab in an ice box.

**Bacteriological examination:** preparation of food homogenate, dilution and spreading were performed according to ICMSF (1978) and Swanson *et al.* (1992).

**Isolation of *Salmonella* (Vassiliadis *et al.*, 1978):** A quantity of 25 gm of sample was pre-enriched in 225 ml buffered peptone water 1% and was incubated at 37 °C for 24 h. Then one ml of pre-enriched broth was transferred into a tube containing 10 ml of Rappaports Vassiliadis (RV) (Oxoid CM669) broth and incubated at 40°C for 48 h. A loopful from the enriched broth was streaked on Xylose-Lysine Desoxycholate (XLD) agar selective medium (Oxoid) and MacConkey agar plates (Oxoid No3); two plates for each sample. The plates were incubated at 37 °C for 24 h. On XLD the red colonies with or without black centres were suspected to be *Salmonella*, so did clear transparent colonies on MacConkey agar. Suspected colonies were cultured on nutrient agar slope for further identification.

**Identification of the isolated *Salmonella*:** a) **Morphological examination:** Films were made from the pure culture of the isolated organism. Stained with Gram's stain and examined under the oil emersion lens; *Salmonella* are Gram-negative short bacilli. b) **Biochemical reaction:** Suspected isolates were biochemically identified to be *Salmonella* using biochemical reactions (TSI), Indole production, Voges-Proskauer and Sugar fermentation. These tests were performed in the serology unit of the Animal Research Institute. c) **Serological identification of *Salmonella* species:** Determination of O (somatic antigens) Separate O antisera (Behringwerke AG Diagnostica) were applied to determine the group of the *Salmonella* isolates. Determination of H (flagellar antigens) Polyvalent H antisera (Behringwerke AG Diagnostica) for both phase 1 and phase 2 were tried in order to determine the complete antigenic formula of the isolates. For these purpose rapid diagnostic H antisera, sets (Behringwerke AG Diagnostica) were used.

From the biochemical reactions, the suspected colonies were subjected to serological identification according to Kauffman white schemes (Kauffman, 1973). The isolates were sub- cultured on nutrient agar plates for 24 h at 37 °C for application of slide agglutination technique. Two separate drops from microbial suspension in physiological saline were applied on clean glass slide. A drop of each of separates O and H *Salmonella* antigens standard loop were added and 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 flakes.

**Preparation of gamma –irradiated *S. typhimurium* vaccine (Xu *et al.*, 2007):** 1- **Preparation of dense cultures of *Salmonella typhimurium*:** *Salmonella typhimurium* recovered from the meat of diarrheic buffalo calves was used to prepare inactive whole cell vaccine. An overnight culture of *S. typhimurium* grown in shaker water bath at 200 rpm in 200 ml of Luria broth. The broth culture was centrifuged at 9000 rpm for 15 min. at room temperature and pellet washed three times with sterile phosphate buffered saline (PBS). The final pellet was resuspended in 50 ml sterile PBS equivalent to a bacterial count of  $1.0 \times 10^{10}$  CFU / ml and diluted by using McFland tube to  $9 \times 10^8$  CFU/ ml. The count was confirmed by using drop plate technique (Fierer and Guiney, 2001).

2- **Irradiation process:** Several trials were carried out to detect the optimum dose of gamma radiation required for complete inactivation of *Salmonella typhimurium*. The microorganism was dispensed into three bottles. The bottles were exposed to gamma rays emitted from cobalt 60 source at room temperature; at a dose rate of 5.6 KGy/hr. The irradiation process was carried out using the facilities of the National Center for radiation Research and Technology, Egypt. After treatment with gamma radiation at doses of 3, 3.5 and 4 kGy, one loopful from each bottle was cultivated on nutrient agar to determine the optimal inactivation dose.

**Quality control of the prepared vaccine:** The *S.typhimurium* was inactivated by 3 KGy dose of gamma irradiation. The vaccine was prepared by mixing  $9 \times 10^8$  CFU /ml of irradiated *Salmonella* combined with Freund's incomplete oil adjuvant. The prepared vaccine was tested for inactivation, purity, sterility and safety according to the standard international protocols described by British Veterinary Codex (1970) and code of the American Federal Regulation (1985).

**Purity test:** This test was done before gamma inactivation of the used *S. typhimurium*. It was applied to confirm that the used broth culture of *S. typhimurium* did not contain any contamination by other organisms before inactivation. Such purity was detected by inoculation of the broth culture onto nutrient agar and incubation at 37°C for 48 hours. Pure colonies of *S.typhimurium* were observed.

**Completion of inactivation for *S.typhimurium*:** In assurance that the used *S. typhimurium* was completely inactivated, nutrient agar plates were inoculated with the irradiated vaccine. After 24-48 hours post inoculation, the detection of complete inactivation was recorded when no visible growth was reported.

**Sterility test:** The prepared vaccine was confirmed to be free from any contaminants by inoculation of Thioglycollate broth, nutrient agar and MacConkey agar media and incubation at 37°C for 72 hours. Also inoculation was done on Sabouraud dextrose agar plates that were incubated at 25°C for 7 days. Inoculation was made also on Mycoplasma broth and agar plates and incubated at 37°C for 72 hours and 14 days in 5 % CO<sub>2</sub> simultaneously.

**Challenge procedure after vaccination of rabbits with the prepared vaccine:** Three weeks after boosting, 25 rabbits (A: unvaccinated non immunized control group, B: subcutaneously vaccinated rabbits group with 1ml, C: subcutaneously vaccinated rabbits group with 1.5 ml, D: intramuscularly vaccinated rabbits group with 1ml, and E: intramuscularly vaccinated rabbits group with 1.5 ml).

**Mortality rate, lesion/ scores:** Following challenge, all rabbits were kept under observation daily for 23 days and the mortality rates were recorded. All dead rabbits and some survivors were subjected to postmortem examination for any characteristic lesions. **Re-isolation:** Re-isolation of *S. typhimurium* from meat samples of diarrheic immunized & non immunized rabbits after challenge. (Serology unit, Animal Health Research Institute, Dokki, Giza.

**RESULTS**

**Table 1:** Showed that out of 200 meat samples. Four bacterial isolates were identified as *Salmonella* species with an incidence 2 % identification of isolates was based on cultural, morphological biochemical character and serological examination.

**Table 1:** Incidence of *Salmonella* species recovered from meat of diarrheic calves:

<i>Salmonella</i> isolates	Diseased calves	
	No. of positive samples	% of positive samples
<i>S.typhimurium</i>	2	1
<i>S. dublin</i>	1	0.5
<i>S. anatum</i>	1	0.5
Total	4	2

\*The percentage was calculated according to the total number of examined samples.

\* Number of examined samples 200.

The culturally and biochemically identified *Salmonella* isolates were serotyped using slide agglutination test according to the Kauffmann-White scheme; the results recorded in Table (2) showed that two of *Salmonella* isolates 2 were *S. typhimurium* 1 was *S. dublin* and 1 was *S. anatum* with an incidence 50 %, 25 % and 25 % respectively.

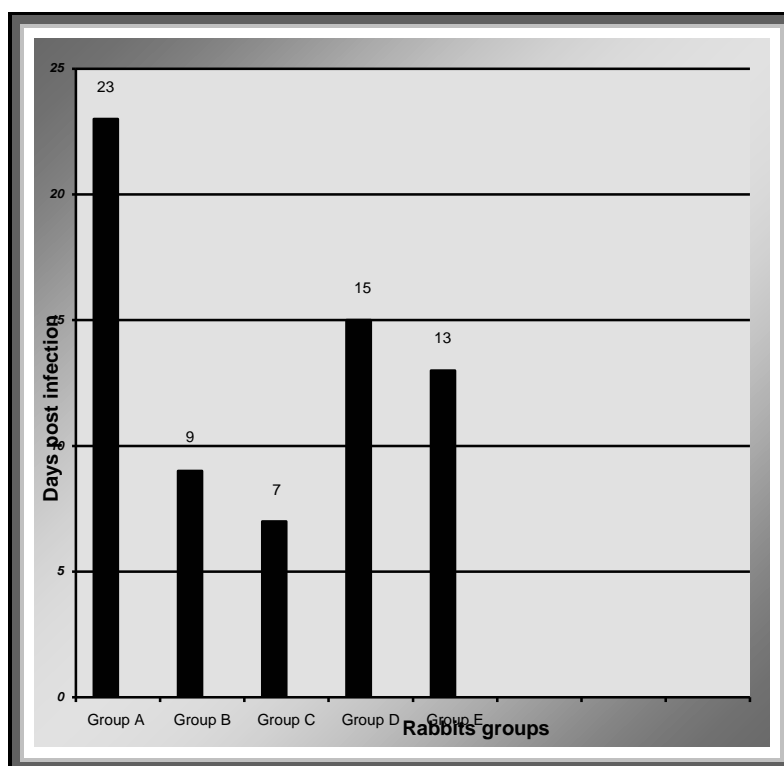
**Table 2:** Antigenic structure of *Salmonella* serovars recovered from 200 meat samples of diarrheic calves:

<i>Salmonella</i> serovars	Antigenic structure	
	O	H
<i>S. typhimurium</i>	1,4,5,12	1,2
<i>S. dublin</i>	1,9,12,Vi	0
<i>S. anatum</i>	3,10	1,6

**Results of irradiation process:** The proper dose of irradiation chosen for the inactivation of the vaccine was 3 KGy for 10 minutes. Results of quality control tests proved that the gamma irradiated inactivated vaccine was confirmed to contain pure cultures of *S. typhimurium* (before gamma irradiation) and the inoculated nutrient agar plates were devoid of any bacterial growth. The sterility test revealed that the vaccine was free from bacterial and fungal

contamination. Also the safety test indicated that the prepared irradiated vaccine was free from any contaminants (aerobic, anaerobic, fungi and Mycoplasma). As regards to safety tests, the prepared vaccines didn't show any abnormalities or adverse reactions during the seven successive days of inoculation of rabbits. While all rabbits were followed out and kept under observation up to the end of the experiment.

Fig. (1) shows that *S. typhimurium* was re-isolated from the faecal samples of rabbits. *S. typhimurium* was shed till 7<sup>th</sup> and 10<sup>th</sup> days then stopped in the subcutaneously immunized rabbits with 1.5 ml and 1 ml respectively. While, in rabbits immunized intramuscularly with 1.5 ml & 1 ml. *Salmonella* still shed to the day 9 and 12 respectively. The control rabbits shed till the day 23.



**Fig. (1): Fecal shedding of *S. typhimurium* in challenged rabbits. Each group= 5 rabbits.**

Rabbits used in the experiment were checked before starting the experiment for *S. typhimurium* infection. Table (3) showed that four rabbits in control group (not vaccinated) died and one lived (20%). One from group B (subcutaneously vaccinated rabbits group with 1ml) died and no rabbits died from group C (subcutaneously vaccinated rabbits group with 1.5 ml) with protection rate of 100%, two rabbits died from group D (intramuscularly vaccinated rabbits group with 1ml); lived rabbits 3 (60%). The last rabbit died with severe symptoms of diarrhoea from group E (intramuscularly vaccinated rabbits group with 1.5 ml); the survivals were 4 (80%).

**Table 3:** Results of challenge test in rabbits vaccinated with irradiated *S. typhimurium* vaccine:

Vaccinated group	Dead/total	Survival/total	Protection rate (%)
A	4/5	1/5	20%
B	1/5	4/5	80 %
C	0/5	5/5	100 %
D	2/5	3/5	60 %
E	1/5	4/5	80 %

Each group= 5 rabbits

## **DISCUSSION**

**The incidence of *Salmonella* species recovered from meat samples:** Three different *Salmonella* serovares were elucidated namely, *S. typhimurium*, *S. anatum* and *S. dublin* with an incidence 1 %, 0.5 %, 0.5 % respectively. Nearly similar results were reported by Fossler *et al.* (2004). While Oloya *et al.* (2007) recorded salmonellosis outbreaks with an incidence 45.7% and 55% respectively.

**Serological identification of the isolates:** The results recorded in Table (2) showed that two of *Salmonella* isolates were *S. typhimurium*, 1 was *S. dublin* and 1 was *S. anatum* with an incidence 50 %, 25 % and 25 % respectively, similar results were reported by Veling *et al.* (2002).

**Irradiation process:** The proper dose of irradiation chosen for the inactivation of the vaccine was 3 KGy as recorded by Alvarez *et al.* (2007); Sangyong *et al.* (2007) and Mohammed, 2010.

The vaccine was found to be safe and produced no clinical symptoms during the seven successive day's observation of the vaccinated rabbits.

**Re-isolation:** *S. typhimurium* was re-isolated from the fecal samples of rabbits. *S. typhimurium* was shed till 7<sup>th</sup> and 10<sup>th</sup> days then stopped in the subcutaneously immunized rabbits with 1.5 ml and 1 ml respectively. While, in rabbits immunized intramuscularly with 1.5 ml & 1 ml *Salmonella* still shed to the day 9 and 12 respectively. The control rabbits shed to the day 23 as shown in Fig. (1). The present results agree to large extent with the observation of (Jonson, 2009) who found that oral infection of rabbits with *S. typhimurium* led to death from 7 to 10 days with symptoms of diarrhea, enteritis and bronchopneumonia, while (Hanes *et al.*, 2001) said that the rabbits began to recover from the infection on day 14 post infection as evidenced by weight gain and increased food consumption by day 23 post infection.

From data available in Table (3), it is evident that the protection percent of the subcutaneously vaccinated rabbits was higher than the protection percent of the intramuscularly vaccinated groups. Meanwhile 100% of unvaccinated rabbits had severe lesions and the mortality percentage was 80%, similar results were recorded by Nemer, 2010. All the inoculated rabbits with viable cells of *S. typhimurium* showed depression, ruffled fur, and anorexia, with yellowish diarrhoea after 2-3 days from infection. Slight watery discharge from eyes and nose was also observed in some rabbits. These results coincide with (Jang *et al.*, 1999) who showed that a higher antibodies response was evoked by subcutaneous immunization because

there was delay in absorbance to vaccine from subcutaneous than intramuscularly.

## **CONCLUSION**

From results obtained in this study; 3KGy of gamma irradiation are enough to eradicate *Salmonella typhimurium* from meat and render the meat safe for consumption also it can be recommended that irradiated vaccine for *S. typhimurium* can be the vaccine of the future. Irradiated vaccine is inexpensive to produce, store, and transport without refrigeration.

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## عزل السالمونيلا من لحم عجول الجاموس وتقييم اولى للقاح المشع منها

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في هذه الدراسة تم الفحص البكتريولوجي ل 200 عينة لحوم من عجول مصابه باعراض معويه وتم عزل السالمونيلا بنسبة 2 % وكذلك تم تصنيف العترات سيرولوجيا باستخدام اجسام مضادة H & O ووجد ان اكثر انواع السالمونيلا عزلا هو السالمونيلا تيفيموريم يليها السالمونيلا اناتم و السالمونيلا دابلن بنسب 1 % و 0.5 % و 0.5 % على التوالي. والغرض من هذه الدراسة هو تحديد جرعة أشعة جاما التي تجعل لحم العجول خالي من ميكروب السالمونيلا وصالح للاستهلاك الادمي وكذلك تقييم الكفاءة المناعية للقاح السالمونيلا المشع عندما يعطى بجرعات وطرق مختلفة في الارانب. ولقد تم تثبيت العترة بالاشعاع لمدة 10 دقائق باستخدام مصدر للكوبلت المشع في درجة حرارة الغرفة عند جرعة 3 كيلو جرابي وقد ثبت ان اللحم كان خاليا من الميكروب بعد التشعيع وقد تم تحضير اللقاح عن طريق مزج 9 x 10<sup>8</sup> وحدة مستعمرة بكتيرية من السالمونيلا المشعة. وباختبار خلو اللقاح المحضر من الملوثات البكتيرية والفطريات. اظهرت النتائج نقاوة وسلامة اللقاح المحضر وان اللقاح خالي من اى ملوثات بكتيرية كما انه امن حيث انه لم يحدث اى اثار جانبية خلال سبعة ايام متتاليه بعد الحقن في الارانب. وباستخدام مجموعه متجانسه من الارانب تم اختبار اللقاح المحضر ولقد تم قياس فاعلية اللقاح المشع عن طريق تحصين 20 ارنب قسموا الى مجموعات كالاتى: المجموعة ب و ج تم تحصينهم تحت الجلد بجرعتين 1 ملم و 1.5 ملم على التوالي. والمجموعة د و ه فقد تم تحصينهم بالحقن العضلي بجرعات 1 ملم و 1.5 ملم على التوالي. اما نتائج اختبار التحدى فقد اظهرت ان اللقاح المحضر يوفر نسبة حماية 80 % و 100 % للارانب المحصنة عن طريق الحقن تحت الجلد بجرعتين 1 ملم و 1.5 ملم بينما الارانب المحصنة بالحقن العضلي فقد حققت نسبة حماية 60 % و 80 % على التوالي. نستخلص من كل هذه النتائج ان اللقاح المشع للسالمونيلا تيفيموريم قد يكون هو لقاح المستقبل ولكن ذلك يتطلب دراسات مكثفة لتقييم استجابة المناعية.