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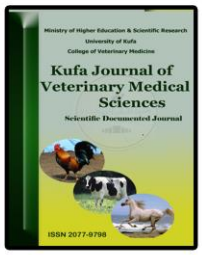

Isolation and Identification of *Salmonella enteritidis* using bacteriological and molecular technique from calves with diarrhea in Diwanihyia city

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Abstract

Salmonella enteritidis is an important foodborne pathogen that affects the human health by conception animal products and it was considered as a worldwide public health hazard. This study was conduct to investigated the prevalence of *S. enteritidis*, in calves with diarrhea in farm of AL- Diwanihyia city basis of molecular properties of this bacterium . A total of 91 samples were collected from December 2013 to May 2014, in different farms in the al Diwanihyia city . the pathogen was isolated by using microbiological and biochemical tests. the DNA extraction was done by genomic DNA kit according to the manufacturer's instruction (USA) and PCR was performed via the specific primers of *SefA- -F* and *SeA-R* of the *SefA* gene. Amplified fragments of the 210 bp were observed in 12 of the total 91 stool samples isolates. This study recommends that the identification of these pathogens by PCR technique can be replaced with traditional bacteriological tools. The PCR method is a rapid approach for recognizing and identifying the *S. enteritidis* infections in diarrheic calves.

Keywords: calves salmonellosis, molecular detection, PCR, foodborne diseases, & epidemiological features.

عزل وتشخيص *salmonella entritidis* من العجول المصابة بالاسهال باستخدام طرق بكتريولوجية وجزيئية في مدينة الديوانية

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الخلاصة

السالمونيلا انترتايتس تعتبر من اهم الممرضات التي تنتقل عبر الاغذية ووالتي تؤثر على صحة الإنسان من خلال المنتجات الحيوانية وتعتبر خطرا على الصحة العامة في جميع أنحاء العالم. وتضمنت هذه الدراسةعزل وتشخيص السالمونيلا انترتايتس باستخدام اختبارات بكتريولوجيه وجزيئية . تم جمع 91 عينة من براز العجول المصابة بالإسهال خلال الفترة من كانون الاول 2013 الى ايار 2014 من حقول مختلفة ضمن مدينة الديوانية , تم عزل الجرثومة في هذه الدراسة باستخدام بعض الاختبارات المكروبيولوجية والبايو كيميائية و تم استخلاص الحامض النووي المنقوص الاوكسجين للجرثومة وتضخيم البادئ البرايمر وفق تعليمات كت الاستخلاص للشركة المصنعة وكانت نتائج التشخيص 12 عزلة وبنسبة (6.18%) من حوالي 91 عينة براز قد اعطت نتائج موجبة لاختبارات العزل والتشخيص لجرثومة السالمونيلا انترتاتيس وتم تأكيد العزل بطريقة بلمرة الحامض النووي الرايبومنقوص الاوكسجين باستخدام البادي *A* gene *Sef* وتضخيم 210 pb . اعتماد طرق التشخيص للبكتريا باستخدام بلمرة الحامض النووي

ال DNA تعتبر حاليا من اهم الطرق البيولوجية واسرعها واكثر تأكيدا للمسبب المرضي من الطرق التقليدية

Introduction:

Salmonellosis represent an important public health problem worldwide. It was estimated that approximately 70%–80% of food borne bacterial outbreaks were caused by *Salmonella* spp. (1, 2). It conceders The most serious infection usually attack calves during the first ten weeks of life (3) and most common host-adapted serotypes involved in bovine Salmonellosis are *Salmonella typhimurium, Salmonella Enteritidis, Salmonella Anatum, Salmonella Newport, *Salmonella Agana*and *Salmonella Dublin* (4, 5). the dairy calves have many impacts on animal and human health that heavy economic losses It was manifested through a number of perdition and poor growth of infected animals as well as the potential of zoonotic transmission (6). Outbreaks with high prevalence of clinical and subclinical *Salmonella* infections have been reported in all parts of the world in cattle & calves, encountered, many isolated seroivars that considered host-adapted *Salmonella* for cattle (7). Foods of animal origin are frequently implicated in human salmonellosis owing to the high prevalence of S*almonella* strains in animals (8). During the last 20 year , it has been a major causative agent of foodborne gastroenteritis in humans (9). In consideration to Salmonella importance as one of the causative agent of human and animal food. poisoning so this study was conduct to investigated the prevalence of *S. enteritidis*, in calves with diarrhea in farm of AL- Diwanihyia city Culturing and isolation of *Salmonella* from fecal samples is needed a development , rapid and sensitive method for the diagnosis of *Salmonella* species is desirable PCR method is rapid, specific and sensitive method for the detection of food borne pathogens (10).

Material &methods :

Sample collection: A total of 91 fecal swabs sample were collected from 194 beef calves suffering from mucoid and/or bloody diarrhea and from apparently healthy contact calves. Calves age ranged from 1 to 6 months. Samples were collected from December 2013 to May 2014, in different farms in the al diwania city. Fecal samples were transferred to the laboratory in a cold chamber container to be cultured without delay.

Isolation of salmonella and identification : *Salmonella isolates* was isolated from fecal samples after inoculated into tetrathionate broth for enrichment for 16 h at 37°C. then A loop full of the broth were streaked onto SS. Agar, XLD agar, plates and incubated at 37°C for 37 - 48 h Biochemical identification was carried out using API 20-E test kit system (Biomeraux, France) Cultivation and identification where applied according to (11).

Primers and PCR Conditions :, Specific Primers Sequence in this study Used for PCR Amplification of S. enterica serovar Eenteritidis *SeFA* gene which encodes salmonella enteritidis fimbria protein produced a DNA fragment of 210 bp. For the PCR assay, This specific primer was designed by using NCBI Gene Bank and Primer: online and provided by (Bioneer company, Korea) as following Table (1-1)**.**

Table (1-1) primer used in this study by using NCBI Gene Bank and Primer:

online and provided by (Bioneer company, Korea)

Primer	sequence		PCR product size
SefA	F	cagccaactggagtcagg at	210bp
	R	tattggctccctgaatacgc	

DNA extraction and DNA Amplification : The bacterial DNA was extracted by using Genomic DNA kit according to the manufacturer's instruction (USA). The amplified DNA products from Salmonella spp. specific-PCR were analyzed with electrophoresis on 1% agarose gel stained with ethidium bromide and visualized by UV illumination depending on DNA marker (1000 bp DNA ladder).

Preparation master mix for PCR : The PCR amplification mixture (20µl) which was used for the detection the *SefA* gene includes 5 µl of (PCR PreMix Lyophilized), which provided by Bioneer (Korea.) include: bacterially derived Taq DNA polymerase; dNTPs which include: 400 µM of each dATP, dGTP, dCTP, dTTP; 3mM of Mgcl2. Yellow and blue dyes as loading dye, 5 µl of template DNA, 1.5 µl of each forwarded and reversed primers and 7. µlpcr water to complete the amplification mixture to 20 µl. The PCR tubes containing an amplification mixture were transferred to thermocycler and started the program for amplication of *SefA* gene .30 cycles of PCR, with 1initial denteration 1 cycle 95C˚ for I min then 5 min at 95C˚ (denaturation), 30 s at 55 C˚ (annealing), and 45s at 72 C˚ (extension), and 1 cycle for 7 min at 72 C˚.

Results &Discussion : The results of bacteriological isolation of *Salmonella .spp* from the collected fecal samples from diarrheic calves revealed the presence of Salmonella organisms in them as shown in (Fig. 1.) All bacteriological positive fecal samples were positive confirmed by PCR and showed amplification of 210bp fragments as shown in (Fig. 2.) *Salmonella entertidis* was isolated from (6.18%) 12 out of 91 fecal samples of calves infected with diarrhea . This identification rate was lower than the reported rates of other studies (12.) who reported % 2.1.The results of isolation *Salmonella entertidis* pathogen according to age groups of infected calves the showed highest isolation rate (6.34% [A]) was in (3- 6) months . while the lowest positivity rate (5.88%[A]) during the ages less than one month. Statistically there was no-significant differences between ages at ($p< 0.05$) as showed in table (1-2). because after birth, calves are directly exposed to contaminated environments which can be influenced by various factors such as the presence of infected animals, overcrowding, concurrent cow-heifer calving, contaminated calving lots, and a lack

of calf segregation by age (13). These factors usually work synergistically and increase the opportunity for increased duration of exposure to a higher quantity of pathogens. Even though Salmonella can cause diarrhea in both adult cattle and calves, infection is much more common and often causes severe symptoms in 10-day to 3-month old calves (14). Calves can shed the organism for variable periods of time and intermittently depending on the degree of infection (e.g., clinical or subclinical infection).

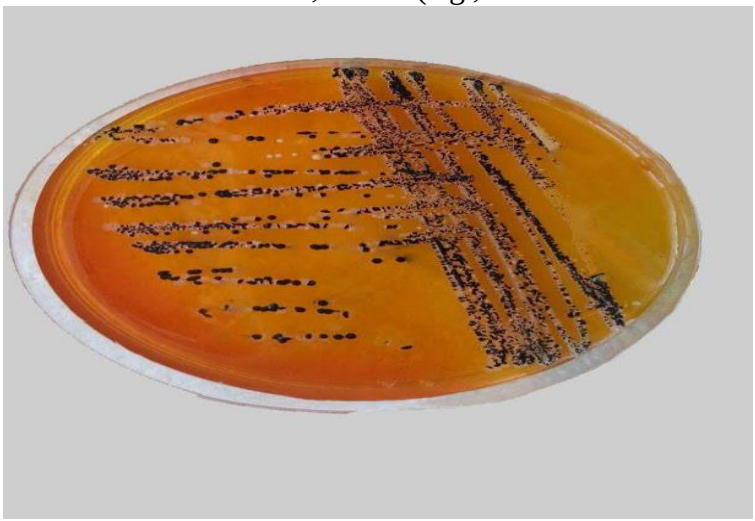

Fig (1) shows isolation *Salmonella .Entertites* on XLD agar

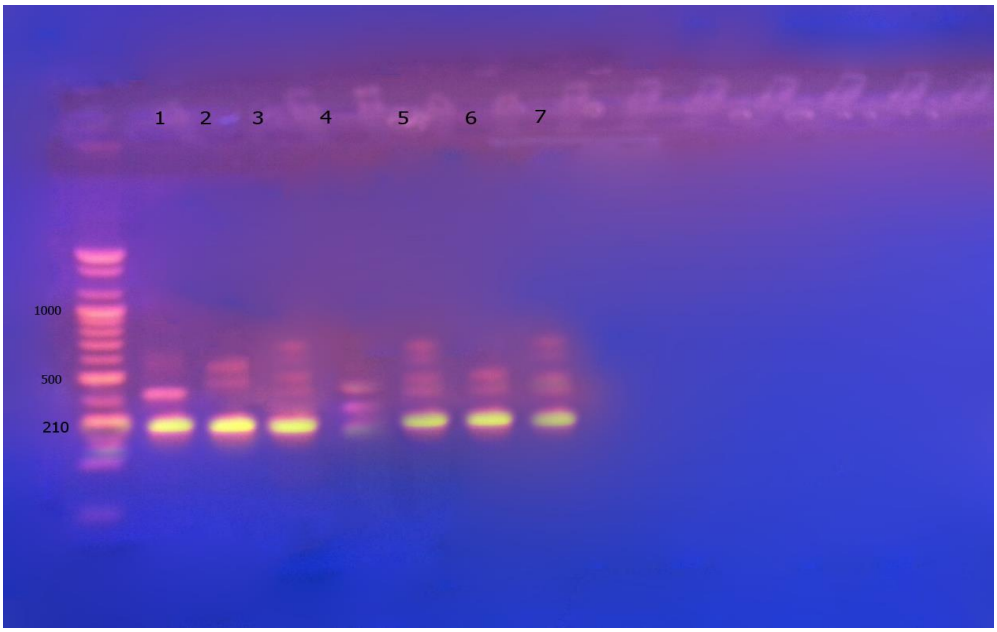

Fig (2) DNA amplification of a 210 bp of *Salmonella entertidis*. detecting *SefA* gene **using singleplex PCR lane 1,2,3,5,6,7 results ,lane,4,5positive results Lane M 1000bp marker (ladder).**

Table (1-2) Results of PCR of *Salmonella entertidis* infection rate according to the calves age groups :

Ages groups	No .of diarrheic samples	No. of positive samples	Positivity Percentage %
1day_1months	68	4	5.88% [A]
(3- 6) months	126	8	6.34% [A]
Total	194	12	6.18%

- **There is no-significant differences between ages group at ($p< 0.05$)**

The results of infection according to calves sex, showed the highest positivity rate (7.89[A] %) between females and the lowest positivity rate in males (3.75[A]%). Statistically, There was no-significant differences between gender at ($p< 0.05$). These results came similar to other Iraqi studies results (15) which indicate that there was an increment in Salmonella isolation rate associated with gender Table (1-3)

Table (1-3) : Results of PCR of *Salmonella entertidis* infection rate according to the calves sex.

Sex	No .of diarrheic samples	No. of positive samples	Positivity Percentage %
Male	80		3.75
Femal	114	9	7.89
Total	194	12	

- **There was non-significant differences between sex at ($p< 0.05$)**

The results of *Salmonella entertidis* infection according to the months December, January, February, March, April and May were (0[A] %) , (2[AB] %), (4.44[BC]%), (

8.33%[C]), (25% [D]), & (0%[A]) respectively. The highest positivity rate recorded in April (25% [D]), while there was no incidence of infection during December & May. Statically there was significant differences between months at ($p< 0.05$). the rate of isolation was appear in this study more likely to occur in the colder months of the year & disappeared in another. these differences are not entirely known. May be related to low level of special care which is required to reduce environmental risk factors closely associated with calving season including the provision of dry, draft-free shelter. The calving season can be adjusted to a time when environmental conditions are more favorable by implementing a controlled breeding program. Exposure to a contaminated environment is the main cause of calf diarrhea Table (1-4).

Table (1-4) Results of PCR of *Salmonella entertidis* according to months

Months	No .of diarrheic sample	No. of positive samples	Positivity Percentage %
December	18	0	0%[A]
January	50	1	2%[AB]
February	45	2	4.44%[B C]
March	48	4	8.33%[C]
April	20	5	25%[D]
May	13	0	0%[A]
Total	194	12	6.18%

- **There was significant differences between months at ($p< 0.05$)**

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