Using of molecular biology techniques compared with conventional detection methods for detection of salmonella in cattle in Egypt

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Abstract A total of 179 samples of fecal swabs from diarrhea suffering cattle and were randomly collected from slaughterhouses as well as dairy farms. The conventional cultural, biochemical and serological methods for detection and identification of *Salmonella* in fecal swabs were applied and the results were compared with those obtained by molecular screening assays using conventional PCR and Real-Time PCR techniques. The obtained results revealed that 9.1% of fecal swabs were positive for *Salmonella* species using conventional cultured methods while 11.8% by using conventional PCR were found to be positive for *Salmonella* species and 15.5% by using Real-Time PCR were found to be positive for *Salmonella* species. The results of the three methods were compared to each others. We conclude that the Real-Time PCR assay has highest sensitivity and specificity for detection of *Salmonella* species in dairy farms especially with large number of samples.
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1. Introduction

Salmonellae are the most complex of all the Enterobacteriaceae with more than 2399 serovars with broad host range, including human, animals and birds.(1)**.** Salmonellae cause avariety of disease manifestations that include acute and chronic enteritis, septicaemia, poly-arthritis, nervous manifestations, death and agalactia in lactating animals with prolonged carriage by survivors(2) Gastroenteritis is associated with mature animals, while septicemia is prevalent among young animals. (3). salmonella produce a variety of putative virulence determinants, including haemaglutinins, adhesions, invasions, fimbriae, exotoxins and endo toxins (4) Salmonella-infected animals in dairy herds and feed lots, must be quickly identified so that they can be isolated from other animals to prevent lateral spread of infection through the fecal-oral route and result in environmental contamination and the eruption of mortal outbreaks (5)**.**

Clinical diagnosis of Salmonellosis is difficult in the living animals due to the non-specific nature of the clinical symptoms. Provisional diagnosis has to be confirmed by the isolation and identification of the etiological organism. Traditionally, the isolation and identification of salmonella infections still rely on conventional bacteriological culture methods to detect the organism in fecal samples(6).

However, culture-based methods for Salmonella diagnosis are laborious and time-consuming, taking up to 7 days to complete, and are inefficient for epidemiological studies in populations with low prevalence of Salmonella (6)

Molecular biological technology has offered powerful tools, namely Polymerase Chain Reaction (PCR) and real-time PCR, for the detection of salmonella-specific DNA in clinical and environmental samples (7)The PCR assay has shown merits as a rapid, sensitive and inexpensive method for the detection of Salmonella in fecal samples. Moreover, PCR has reduced the time required for the accurate detection of salmonellae in clinical samples to 12 to 20 h, thus enhancing the efficiency of epidemiological studies of Salmonella. (6, 8)

The invA gene is the most accepted target of PCR assays because it is not only specific to the Salmonella genus, but it is also found in all known serovars of Salmonella (6, 9)**.**

The aim of the present research is Isolation, identification and serotyping of salmonella organisms from the faeces of diarrheic calves, using conventional bacteriological methods and Molecular detection of salmonella organisms in the faecal samples of diarrheic calves using conventional PCR assay, as well as the real-time PCR assay, targeting the invA gene. Finally comparison between the results obtained by these techniques.

2. Martial and Methods

Samples:-

A total of 179 fecal swaps and bile samples were collected from slaughterhouse (32 colon contents and 36 bile samples) as in table 1 as well as 6 dairy farms (110 fecal swaps and 1 bile samples) as in table 2.

Slaughter house samples were collected from Baladi cows and feedlot calves. Animals were apparently healthy with no diarrhea. While samples from dairy farms were from calves suffering diarrhea (1-3 months) and one bile samples was collected from a moribund calf.

Table 1: Slaughterhouse Samples

Samples	**Numbers**
Colon contents	32
Bile	36
Total	**68**

Table 2: Samples from dairy farms

Samples	**Fecal Swaps**	**Bile**
Farm 1	20	--
Farm 2	22	--
Farm 3	6	--
Farm 4	3	--
Farm 5	15	1
Farm 6	44	--
Total	**110**	**1**

Isolation and biochemical identification of salmonella:

Fecal swaps and bile samples were cultured in selenite F broth then incubated at 37 ^{0}c for 18 hours. loopful from inoculated broth was streaked onto the surface of S.S. agar plates, then incubated at 37 ^{0}c for 24-48 hours. Colonies were identified culturally and biochemically according to Collier *et al*. (10)

DNA extraction and PCR amplification

Genomic DNA was extracted from fecal swaps using TIANamp Stool DNA Kit instructions according to manufacturer's manual. The obtained DNA was stored at -20 °C until used in the downstream applications. PCR technique was applied using primers developed by Swamy et al. (11) that specifically detect 521bp from invA gene of salmonella spp. The assay utilized **invA sense** primer with a sequence 5- TTGTTACGGCTATTTTGACCA -3 and **invA anti sense primer** with a sequence 5- CTGACTGCTACCTTGCTGATG -3. PCR was performed according to Swamy *et al*. (11). The thermal profile was 94^{0}C for 5 min, followed by 35 cycles repeated of denaturation at 94^{0}C for 1 min, annealing at 62^{0}C for 1 min, and extension at 72^{0}C for 1 min. then a final extension at 72^{0}C for 5 min, after that holding stage at 4^{0}C for infinite time. 8 µl of the generated PCR products were migrated on 1.5 % ethidium bromide stained agarose gel under constant volt of 80 V for 40 min. The gel was then visualized using UV- Transilluminator then photographed by the associated camera (12). *Salmonella* positive samples showed a band of 521 bp.

Real-time PCR primers and TaqMan probe:

For the real-time PCR assay, two primers from invA gene, forwared primer (5- GCGTTCTGAACCTTTGGTAATAA -

3) and reverse primer (5- CGTTCGGGCAATTCGTTA -

3). The fluorescent Taq Man probe (5′-FAM-TGGCGGTGGGTTTTGTTGTCTTCT-TAMRA-3′) (13)

Real-time PCR assay

For the real-time PCR assay, a PCR mixture consisting of 7 µl of DNA template, 12.5 µl of 2x QuantiTect Probe RT-PCR Master Mix, 4.375 µl of nuclease free water 0.5 µl of each primer, and 0.125 µl of the TaqMan probe was prepared in 25 µl volume. The real-time PCR was performed in a 96-well optical plate (Stratagene MX3005P) under the following conditions: 5 min at 94 C to achieve optimal enzyme activity, then 40 cycles of 30 s at 95 C and 30 s at 49 ^{0}C. according to Daum et al (13)**.**

3. Results

Isolation and biochemical identification of *Salmonella* spp.

It is noteworthy that samples of colon contents and bile samples collected from slaughterhouse, comprising 68 samples from apparently healthy cows and feedlot calves, did not yield Salmonella organisms upon culture. However, salmonella organisms were readily isolated from faecal swaps collected from calves of dairy farms suffering diarrhea. Out of 110 faecal swaps from diarrheic calves (1-3 months), 10 salmonella isolates were isolated representing 9.1% (Table 3).

Table 3. Prevalence of *Salmonella* isolates recovered from calves suffering diarrhea

Samples	**Fecal Swaps**	**Salmonella isolates**
Farm 1	20	1
Farm 2	22	1
Farm 3	6	1
Farm 4	3	1
Farm 5	15	2
Farm 6	44	4
Total	**110**	**10 (9.1%)**

Suspected colonies for Salmonella appeared on SS agar as transparent colonies with black center as shown in photo (1). for biochemical identification, *Salmonella* isolates were all positive for citrate utilization, methyl red, hydrogen sulphide on TSI.

They were negative for indole production, Voges Prauskuer, lactose fermentation and urea hydrolysis tests.

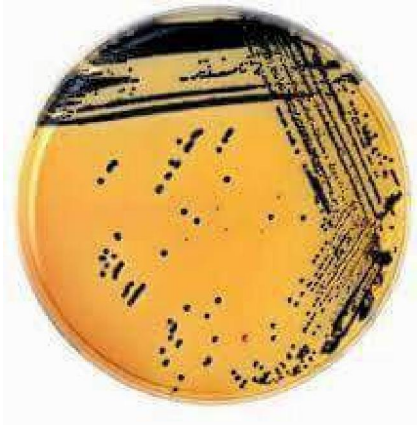

Figure (1): Growth of salmonella colonies on SS agar medium

PCR for Molecular Identification of Salmonella isolates

Salmonella isolates were subjected to molecular identification through amplification of the ***invA*** gene specific for invasiveness of Salmonella spp.

All isolates proved to be salmonella spp., as ***invA*** gene was detected in the genome of all tested isolates through PCR (Figure2). Prevalence of salmonella by PCR 13 (11.8%). including all 10 positive culture and other 3 negatives so PCR proved to be more sensitive than conventional culture methods.

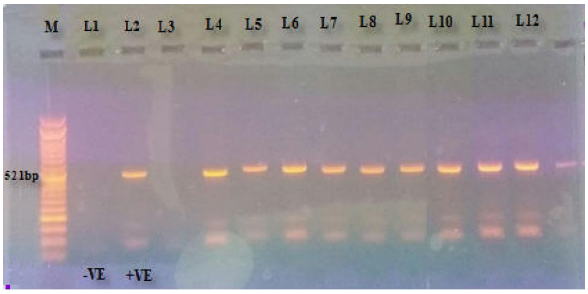

Figure 2. Agarose gel electrophoresis showing PCR results for detection of *invA* gene specific for Salmonella spp. L1 control negative DNA, L2 control positive DNA, L3 control negative sample. Lanes L4 to L13 isolates positive for *invA*. M DNA marker

Detection of Salmonella spp. In Faecal Samples of Diarrheic calves using Real-Time PCR

As a pilot study, a total of 44 faecal samples from a dairy farm suffering sever calf enteritis with calf fatalities were tested for Salmonellosis by the real time PCR technique, using primers for the invasion gene *invA*. As illustrated in figure 3.

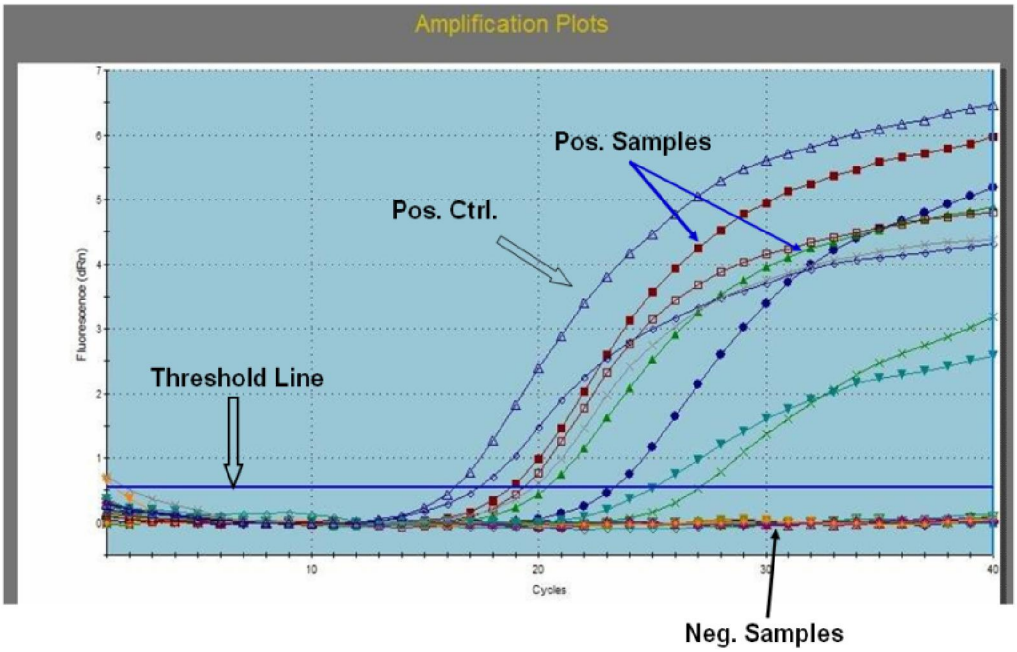

Figure 4. Amplification plots of Real Time PCR of genomic DNA isolated from faecal samples of diarrheic calves of a dairy farm. The primers represent the *invA* gene of Salmonellae. The figure illustrates the Threshold Line, the Control Positive DNA and 8 positive samples (all above the threshold line), and the Negative Samples below the Threshold line.

Comparison between the 3 diagnostic techniques showed that the highest sensitivity is for real-time PCR followed by conventional pcr then bacteriological culture as illustrated in (Table 4).

Table 4. Results of direct bacteriological isolation, Conventional PCR and Real-Time PCR for the detection of *Salmonella* in faeces of calves suffering diarrhea

Samples	Fecal Swaps	Salmonella isolates	PCR	RT-PCR
Farm 1	20	1	2	3
Farm 2	22	1	2	2
Farm 3	6	1	1	1
Farm 4	3	1	1	1
Farm 5	15	2	2	2
Farm 6	44	4	5	8
Total	**110**	**10**	**13**	**17**
%		**9.1**	**11.8**	**15.5**

4. Discussion

The present study was conducted to shed light on *Salmonella* species isolated from fecal swaps. in the present study *salmonella* species were isolated by 9.1% on SS agar from fecal samples that is collected from calves suffering from diarrhea. This agreed with Addis *et al*. (14) in Addis Ababa collected milk and fecal samples from dairy cows and their results were agreed with our results that was 10.7% (21/195). Also Gwida *et al*. (15) in Egypt recorded 12% prevalence of *salmonella spp*. In dairy products by culture methods Our results are higher than results obtained by Mcevoy *et al*. (16) that collected samples from abattoir over 12 month period from beef calves and *salmonella spp*. was isolated from 2% of faecal samples and 2% of rumen contents. Also in the United States (17) also determined the prevalence by culture (2.3%). This may be due to higher hygienic measures in these study areas. In contrast in Omdurman locality in Sudan (18) isolated *salmonella spp*. By higher prevalence that is reached to 40% from fecal samples, and this indicated poor hygienic measures in dairy farms in this locality.

Our *Salmonella* isolates were subjected to molecular identification through amplification of the ***invA*** gene specific for invasiveness of *Salmonella spp*. And proved to be *salmonella spp*. With prevalence 11.8% And PCR appeared to have higher sensitivity than conventional culture methods. this was in agree with Gwida *et al*. (15) Eriksson et al. (19) Kwang *et al* (20). that recorded higher sensitivity for PCR than culture method.

In present study by application of Real-Time PCR on *Salmonella*, the prevalence was 17 samples (15.5%) with higher sensitivity than conventional PCR. And this was the most sensitive method for diagnosis of salmonella spp. Researches that applied on Real-Time PCR on *salmonella spp*. Proved highest sensitivity and specificity (21, 22).

About comparison between the three diagnostic techniques our results showed that Real Time PCR has the highest sensitivity followed by conventional PCR then conventional culture methods and this was in agree with Gwida et al. (15) Eriksson *et al*. (19) Wilkins *et al*. (21).

We concluded that Real-Time PCR was most beneficial specially when large numbers of samples have to be examined as it has highest sensitivity and time consuming than conventional PCR and conventional isolation methods.

Conclusion:

The above study has a final conclusion of proving the advantages of molecular biology diagnostic techniques in the direct diagnosis and detection of salmonella species infection in diarrhea suffering cattle in Egypt. This study clearly revealed the higher sensitivity of conventional polymerase chain reaction (Conventional PCR) compared with conventional cultural detection and identification methods, and also showed the higher sensitivity and efficacy of Real-Time polymerase chain reaction (R-T. PCR) compared with both conventional polymerase chain reaction (Conventional PCR) and conventional culture and identification techniques.

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