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COMPARISON OF PCR AMPLIFICATION OF THE *FIM A* GENE AND CULTURAL METHOD FOR DETECTION OF SALMONELLAE IN FEED AND WATER

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SUMMARY

Bacteriological examination of local and imported feedstuff samples revealed that 11 out of 600 feedstuff samples harboured salmonellae with an incidence 1.8%. The highest incidence of isolation was in imported bone and meat meal (4%). A polymerase chain reaction was performed by amplification *fim A* gene as specific method for detection of Salmonella in feed and water. In order to obtain higher specificity, we have selected a series of primers internal to the *fim A* gene sequence. The concordance percent between conventional cultural method and PCR of feed and water were 96.6% and 100% respectively. This assay enable to detect 10 C.F.U./ 100 ml water and 10 C.F.U./ 25 g feedstuffs and it could detected only Salmonella strains. The detection of all positive samples and the failure to amplify the fragment from non Salmonella strains confirm that the *fim A* gene contain sequences unique to Salmonella genus and demonstrate that this gene is suitable PCR target for detection of Salmonella bacteria. This rapid assay provides a sensitive and specific means of screening drinking water samples as well as feedstuff samples for the presence of Salmonella spp.

INTRODUCTION

Salmonellae are one of the most important microorganisms that cause disease to man and animals and among the most common causes implicated in outbreaks of food born infectious disease around the world, (Baird-parker, 1990) and (Lacey, 1993). Animals are mainly infected through feed, drinking water or environmental sources, (Soumet, 1999). The risk of Salmonella infection has been hightened by the globalization of trade in food, feed and live animal and changes in production, processing and handling of foods (Waage at al., 1999). Individuals infected

with Salmonella shed the organism in their feces, which enter the domestic sewege that, in turn, may contaminate drinking water sources. Although the concentration of Salmonella in water is low, ingestion of water can still cause of infection, because the water may pass rapidly through the stomach into the intestine without stimulating digestion and thereby escape the natural host defence mechanism, (Murray, 1991). To determine Salmonella in feedstuffs and water sources sensitive and specific detection methods are needed. There are several problems concerning the detection of Salmonella in feed and water such as their low numbers and intermittent presence.

Traditional methods used for the detection and isolation of salmonellae from feedstuffs and water have been based on culture of the organisms. These cultural method are laborous, insensitive and time consuming. To overcome these drawbacks immunological and DNA methods have been developed among which PCR has been applied.

The use of PCR technique widely adopted during the last few years as it provides new strategy for rapid and sensitive detection of salmonellae. PCR has been shown to allow the detection of single cell ,(Li et al., 1988). Several PCR assays for detection of salmonellae have been developed and several different target DNA loci for amplification have been applied, (Cohen et al., 1996 and Waage et al., 1999).

Salmonella typhimurium and other pathogenic members of the family Enterobacteriaceae produce morphologically and antigenically related, thin, aggregative, type 1 fimbriae. A single gene *fim A* encodes the major fimbrial subunit, (Purcell et al., 1987). Although certain types of *E. coli* fimbriae are known to be important for virulence, only type 1 fimbriae have been implicated in Salmonella pathogenicity. *Fim A* gene has been cloned and sequenced, (Nicols et al., 1990 and Swenson and et al., 1994). Primers were designed specifically to amplify a certain region of *fim A* gene from Salmonella strains. The objective of this study was :

1. Isolation and identification of salmonellae from local, imported feedstuffs and water by conventional cultural methods.
2. Detection of salmonellae in feedstuffs and water by PCR.
3. Comparative study between cultural method and PCR.
4. Determination of PCR specificity by inoculating *S. typhimurium* and other entric bacteria in feed stuffs.
5. Determination of PCR sensitivity by inoculating *S. typhimurium* in different concentrations in feed stuffs and water.

MATERIAL AND METHODS

Samples:

* A total of 600 feedstuff samples and 50 water samples were examined. They include 175 im-

ported and 425 local feedstuffs. Samples were collected from different areas of Egypt.

Each feed sample was collected in sterile polyethylene plastic bag while water was collected in sterile glass bottle from water supply and well water.

*** Strains:**

A total of 4 stains of *S. typhimurium* LT2 , *E. coli*, Shigella spp., *K. pneumoniae* were obtained from animal health research institute.

*** Isolation of salmonellae by conventional cultural method:**

A. In feed: According to the procedures of the International Commission on Microbiological Specification for Food "I.C.M. S. F." (1978).

B. In water: According to Standard methods for Examination of water and waste water, "A.P.H.A." (1992). The suspected colonies were described for their morphological and characteristic appearance. Pure cultures of the isolates were identified biochemically according to Barbara et al. (1994). Serological identification of suspected Salmonella isolates were carried out according to the Kauffamann white scheme as described by Kauffmann (1973).

***Detection of salmonellae by PCR:**

Preparation of samples for PCR

In feed: according to Cohen et al. (1996):

- 100 gm of each feed sample was mixed with 200 ml of buffered peptone water and incubated for 1 hr at 37°C then 700 ml of peptone water was added to mixture and incubate at 37 °C for 24 hr.
- 1 ml of incubated broth was added to 9 ml of tetrathionate broth and incubated at 37°C for 24 hr.
- Samples were centrifuged at 9000 r.p.m. for 15 minutes.
- A loopfull of pellet from each sample was transferred to test tube containing 2 gm of glass beads and 50 µl of 10% tween 80 vortexed for 30 second then add 2 ml of 0.25 M NaCl - 1 mM EDTA.
- Two ml of phenol - chloroform - isoamyl alcohol (25: 24: 1) was add and vortexed.
- Centrifuged at 9000 r.p.m. for 20 minutes.
- Supernatant was carefully transferred to another tube then add phenol chloroform mixture (to extract DNA).
- To precipitate DNA, 3 M sodium acetate and ethanol was added then resuspended in Tris-Hcl (pH 7.4).

*** In water: according to Waage et al. (1999).**

- Each sample was filtered through 0.45 µm pore size standerd membrane filter using a vaccum pump.
- Filter was transferred to test tubes containing 10 ml trypton soya broth and incubate at 37°C for 24 hr. with gentle shaking (using shaker).
- 100 µl of incubated broth were transferred to Eppendorf tubes and centrifuged at 9000 r.p.m. for 10 minutes.

-Resuspend the resulting pellets in 50 µl. PCR buffer with proteinase K (0.2 mg ml) and incubate at 37°C for 1 hr.

- Boiling for 10 minutes (to lyse the bacteria)
- Store the samples at - 20°C over night before PCR.
- The samples were thowed at room temperature and were centrifuged at 9000 r.p.m. for 5 minutes. To 50 µl of supernatant fluid add 50 µl of PCR reagent, then the PCR was done.

*** Sequence of used primers:**

- The primers were selected on basis of sequence of the *fim A* gene (EMBL and Genbank accession number M 18283 and sequence name F2 M47) of *S. typhimurium* LT2. The primers were obtained from MWG biotech AG, Germany.
- The 20-mer forward primer (*fim 1 A*), 5' - CCT, TTC, TCC, ATC, GTC, CTG and AA-3' has a calculated temperature of 60°C and is located between 586 and 605 on the *fim A* gene of *S. typhimurium* LT2. The 20-mer reverse primer (*Fim 2 A*), 5'-TGG, TGT, TAT, CTG, CCT, GAC and CA-3' has a calculated annealing temperature of 60°C and is located between bp 651 and 670 on the *fim A* gene of *S. typhimurium* LT2. An 85 -bp fragmet was specifically using this set of primes.

PCR Technique:

PCR Technique was done in biotechnology center - faculty of Veterinary Medicine Cairo University. Amplification of the *fim A* gene was achieved in thermocycler as follows:

- 20 cycles of PCR with 1 cycle consisting of 1 minute at 94°C (denaturation), 30 seconds at 58 °C ,(annealing) and 1 minute at 72°C (primer extension).
- An additional cycle of 1 minute at 94°C , 30 second at 58°C, and 5 minutes at 72°C.
- A 25 µl PCR mixture contain the following: 50 m M tris Hcl and 200 µM of each dATP, dCTP, dGTP and dTTP, 0.075 µM of each primer, 0.65 U of ampli Taq (perkin-elmer) and 2.5 mM MgCl2 Genomic DNA at concentration of 4 ng/µl or a single colony were used in the PCR mixture. *S. typhimurium* LT2 was used as a positive control and reagent without DNA was used as a negative control to monitor contamination of the PCR reagents.

*** Polyacrylamide gel analysis (PAGE):**

-Sheets of 10% polyacrylamide with 10% glycerol 0.75 mm thick were prepared in minprotean II polyacrylamide gel electrophoresis.

- 0.5 x TBE (Tris borate - EDTA) was used as a running buffer.
- The gels were run at 155 v for 45 minutes. The gels were stained with ethidium bromide (10 µg/ml) in TBE and photographed under UV with a Polaroid camera.

-A DNA size ladder ranging from 72 to 1353 bp was used as marker.

*** Correlation of the results of PCR and con-**

ventional cultural method in term of concordance, Ruppanet et al. (1980).

Concordance % =

$$\frac{\text{No. of positive in both tests + No. of negative in both tests}}{\text{No. of samples}} \times 100$$

*** Specificity of PCR :**

To ensure that PCR amplify only Salmonella strains and not other enteric microorganisms. Single colony from *S. typhimurium* and other Gm -ve bacteria including *E. coli*, Shigella spp. and *K. pneumoniae* were tested.

*** Sensitivity of amplification:**

Sensitivity is the ability of the PCR to detect the *fim A* gene in the highest bacterial dilutions. Serial dilutions of *S. typhimurium* 10^1, 10^2, 10^3 C.F.U./100 ml water and 10^1, 10^2, 10^3 C.F.U./25 g feed stuffs were inoculated in sterile feed and water. Then examined by PCR after preparation as mentioned before.

RESULTS AND DISCUSSION

Table (1) & (2) showed the incidence of salmonellae in feed stuffs either of local or imported or-

Table (1): Incidence of salmonellae in local feed stuffs and their serovars.

Type of examined local samples	No. of examined samples	No. of positive samples	%*	Isolated serovars
Concentrates	100	0	0	-
Bone and meat meal	75	2	2.7	*S.typhimurium* *S.montevideo*
Bone meal	75	2	2.7	*S.banana* *S.montevideo*
Poultry residues	75	1	1.3	*S.Reubeuss*
Fish meal	50	1	2	*S.Virshow*
Corn	50	0	0	-
Total	425	6	1.4**	

* percentage was calculated according to number of each type of examined samples.
** Percentage was calculated according to total number of local samples.

Table (2): Incidence of salmonellae in imported feed stuffs and their serovars.

Type of examined imported samples	No. of examined samples	No. of positive samples	%*	Isolated serovars
Bone and meat meal	100	4	4	*S.boecker* *S.Livingstone* *S.Cerro* *S.virshow*
Fish meal	75	1	1.3	*S.agona*
Total	175	5	2.85**	

* percentage was calculated according to number of each type of examined samples.
** Percentage was calculated according to total number of imported samples.

igins.

Results revealed that 11 out of 600 feedstuff samples harboured various salmonellae with an incidence 1.8% and the incidence of salmonellae in local and imported feedstuffs were 1.4% and 2.85%, respectively. The highest incidence of isolation was detected in imported bone and meat meal (4%) that harboured 4 serovars (*S. boecker, S. livingstone, S. cerro* and *S. virshow*), followed by local bone and meat meal and bone meal (2.7%) each. These results coinciding with (Davies et al., 1997 and El-Sayed, 1997) and Lower than (Ebrahim, 2000) who reported that incidence of salmonellae in local bone and meat meal was 4%. *S. typhimurium* and *S. montevideo* were recovered from bone and meat meal while bone meal harboured *S. banana* and *S. montevideo.*

It was interst to notice that *S. banana* was recovered for first time from feed in Egypt. *S. virshow*, *S. agona* and *S. reubeuss* were recovered from local fish meal, imported fish meal and poultry residue with an incidence 2%, 1.3% and 1.3%, respectively. Salmonella spp. couldn't be isolated from concentrates and corn.

No clear pattern could be established relative to isolation of specific serovars of salmonellae in relation to specific types of feed or byproducts.
The results of the present study indicated that incidence of salmonellae in imported feedstuffs are higher than local feedstuffs. These results agree with (Ebrahim, 2000). There is variation in percent of Samonella isolation between different kinds of examined feed, these may be due to different origin, methods of conditioning, steaming and heating and post processing contamination.

Table (3): Comparative study between incidence of salmonellae by conventional cultural methods and PCR in feedstuffs.

Conventional cultural method				PCR			
No. of examined samples	No. of positive samples	%	No. of negative samples	No. of examined samples	No. of positive samples	%	No. of negative samples
600	11	1.8%	589	30*	12	40%	18

* Number of examined samples by PCR including positive samples by cultural method and other random selected samples.

Examination of 50 well water samples for salmonellae by conventional cultural method revealed negative results.

Environmental monitoring of microorganisms to detected potential sources of pathogens requires a high degree of sensitivity. PCR combines simplicity with a potential for high specificity and sensitivity in the detection of food born pathogenic bacteria, (Bej et al., 1994).

In this study two primers which specifically amplify an 85 bp fragment were selected completely internal to the *fim A* gene. Designing the primers to amplify only Salmonella DNA with a small fragment make it useful for synthesis on synthesizer for Salmonella detection. Most Salmonella serovars form type 1 fimbriae which bestow on bacteria diverse adhesive properties but strains of *S. gallinarum* and *S. pullorum* form type 2 fimbriae which lack their adhesiveness. (Cohen et al., 1996) stated that *S. gallinarum* yield an amplicon *fim A*, indicating that the gene is still present, which may be inactive form.

Several other PCR methods have been developed by selecting different region of Salmonella genome. (Rahn et al., 1992) amplified an internal fragment of the inv A gene but two Salmonella spp. (*S. linchfield* and *S. senftenberg*) didn't amplify. (Aabo et al., 1993) selected primers from a 2.3 kb Salmonella specific random sequence and amplified a 429 bp fragment by PCR. Though all non Salmonella strains not amplify two strains of *S. arizona* monophasic failed to yield a fragment. (Jun et al., 1999) suggested that the PCR for sef A gene could be useful for diagnosing Salmonella serogroup D1.

As shown in table (3) only 30 feedstuff samples were used for comparison between conventional cultural method and PCR including 11 positive samples by cultural method and other 19 negative

random selected samples. PCR detected only one more Salmonella positive sample than cultural method. This result in accordance with (Dalsgaard and Olsen, 1995) while, (Stone et al., 1995) recorded that 3 samples were positive by PCR more than cultural method.

This positive sample by PCR was initially classified as being false positive or PCR may be, is more sensitive than the cultural method as salmonellae may have been missed because they were present in very low numbers and injured due to heat treatment during processing of feedstuffs or dead cell so couldn't detect by cultural method.

The percent of concordance when comparing conventional cultural method and PCR in feed- was 96.66%. This agree to large extent with (Bennett et al. ,1998, Fach et al. ,1999 and Soumet et al., 1999b) who reported that the

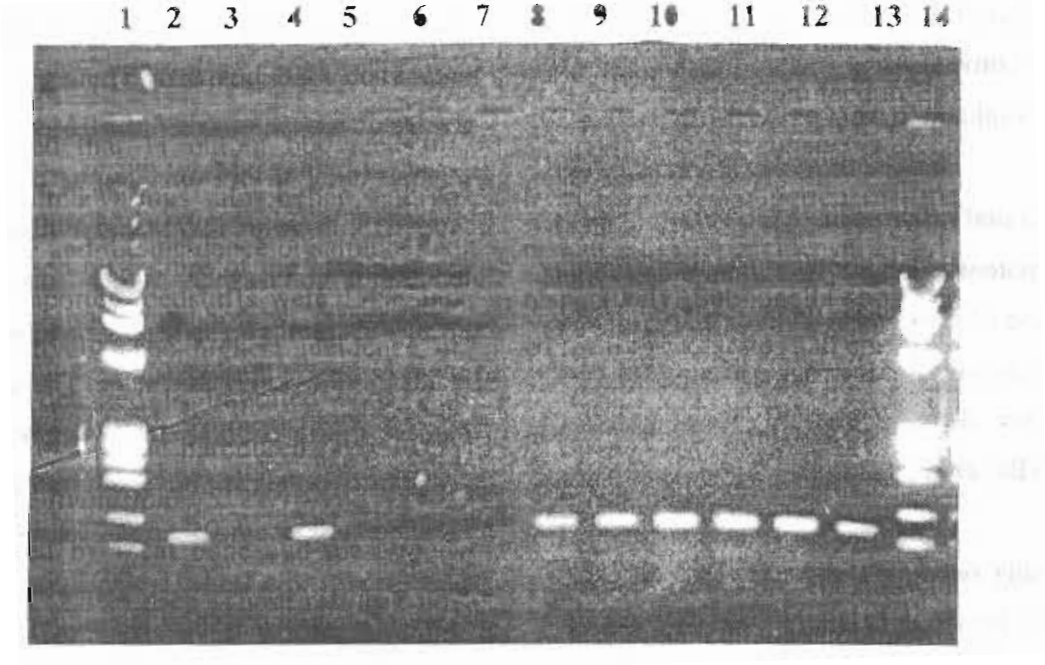

Fig. (1): PCR amplification mixture were run on 10% polyacrylamide gel stained with ethidium bromide: Lanes 1, 14 molecular weight marker [is blac III digest of Ø x 17 µ DNA (Finnzyme fineland) 1353, 1078, ,872, 603, 310, 281, 234, 194, 118 and 72 bp], 2 positive control *S. typhimurium* LT2; 3 negative control no DNA, 4 positive sample; 5 sample was incuolated by *E. coli*, 6 Shigella spp., 7 *K. pneumoniae*, 8-9-10-11-12-13 positive samples of feedstuffs (It lies between 72 and 118 about 85 pb).

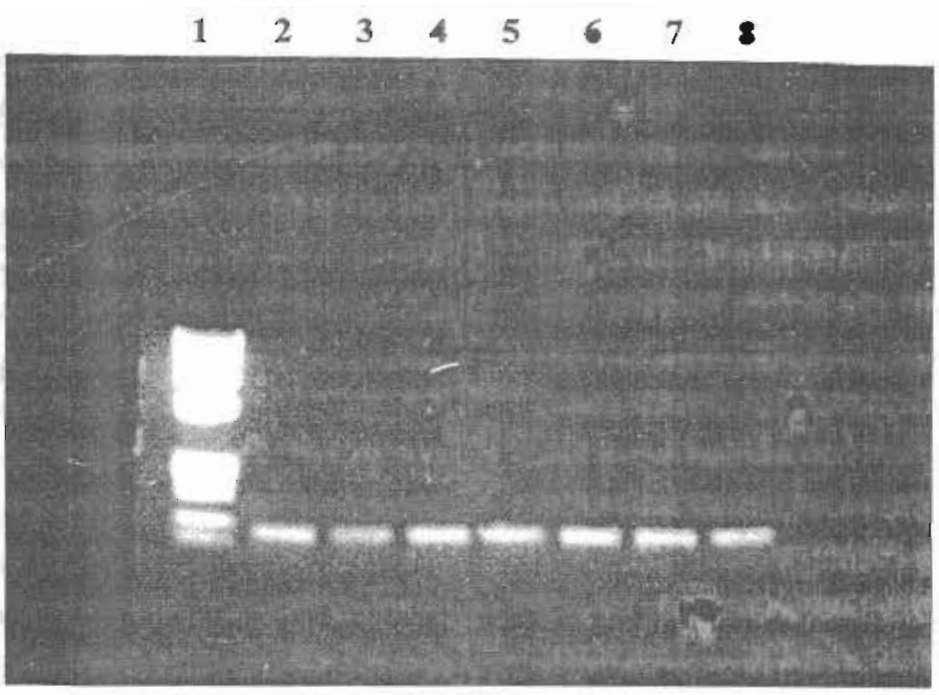

Fig. (2): Sensitivity of PCR amplification, Lanes: 1 molecular weight marker, 2 positive control *S. typhimurium* LT2, 3, 4, 5 samples of feedstuffs were inoculated by *S. typhimurium* 10^1, 10^2 10^3 C.F.U./25 gm feed , 6, 7, 8 water samples were inculated by *S.typhimurium* 10^1, 10^2, 10^3 C.F.U./100 ml. .

agreement percent were 95.6%, 96.6% and 95.6% respectively. On the other hand (Soumet et al., 1999a) recorded 84% agreement between cultural method and PCR.

10 water samples were examined by PCR after preparation of water samples to overcome the major obstacle as humic substance which are strong inhibitor of PCR possibly by interacting with DNA polymerase or with binding of primers to the templatc and therapy greatly reduction the detection limit. The major drawback of using a procedure without enrichment is that the risk of detecting dead bacteria, that obvious are unable to cause infection is increased. The use of non-selective medium such as TSB will increase the chance recovery and growth of stressed Salmonella compared with selective media,(Waage et al. ,1999).

Salmonellae could not be detected in water samples and the concordance percent was 100% with cultural method. This agree with (Coquard et al., 1999) who concluded that the PCR can advantaglly replace the isolation method. In water, it is quicker, less labour intensive, reproducible and provides results that match perfectly to those obtained by isolation method.

Feed and water were inoculated by *S. typhimurium* to detect sensitivity of PCR shown in Fig. (2).

This assay enable to detect all inoculated samples even 10 C.F.U. / 25g feed and 10 C.F.U. / 100ml water. These results coincided to large extent with (Waage et al., 1999) who stated that PCR performed on enriched broth enable detection 10 C.F.U./100 water and < 10 C.F.U./ g in food.

On the other hand, (Maffezzoli et al., 1995) recorded that PCR technique was highly specific and amplified DNA from all tested strains with sensitivity of 1 cell Salmonella /25 gm food while (Fach et al., 1999) found threshold sensitivity was 10^2 C.F.U./ml water, the minimum detection level of dead cells in food samples was superior to 10^6 C.F.U./25 gm.

PCR could discriminate Salmonella from non Salmonella bacteria. The nucleotide sequence of the *fim A* gene of *E. coli, K. pneumoniae* have significant homology but are not fully homologous (Cohen et al., 1996). Primers were designed specifically to amplify regions of the *fim A* gene from Salmonella strains but not from *E. coli* or *K. pneumoniae*. To ensure the specificity of our primers we have taken advantage of the fact that Ampli Taq DNA polymerase doesn't extend primers across its template if there is even a single nucleotide mismatch at the 3'oH ends and we designed oligonucleotide primers to a region where the *S. typhimurium* nucleotide sequence do not match the 3' - ends of the *fim A* genes of *E. coli* and *K. pneumoniae*. By selecting primers completely internal to the *fim A* gene, all non Salmonella strains responded negatively. False positive results were obtained by PCR methods for Shigella spp., (Jones et al., 1993) and verotoxin producing *E. coli*, (Pollard et al., 1994).

The size of the amplicon 85 bp made it a promising diagnostic tool for the sensitivity and specificity. The fim A gene seem to be unique at least in the region of our primers to Salmonella strains and can differentiated between Salmonella and non Salmonella. This is very useful in the diagnosis of Salmonella organisms at the genus level but not at species level. The speed of assay, ease of use and high specificity and sensitivity make it reliable and sensitive technique for use in routine analysis of Salmonella detection in feed and water.

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