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BACTERIOLOGICAL STUDIES ON SALMONELLA TYPHIMURIUM ISOLATED FROM DIFFERENT SOURCES

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SUMMARY

Salmonella enterica serovar Typhimurium isolates recovered from cow calves, lambs and human were tested for their virulence attributes using Congo red binding test, ability to produce hemolysin, adherence assay and HEp2 cell invasion test. Moreover, antimicrobial drug sensitivity was determined. Using disc diffusion method and Genotypic characteristics including the presence of invA gene were studied using PCR. Six isolates were examined by PCR to determine the virulence invA gene which is responsible for invasion property of salmonella strains. The tested isolates were positive for invA gene sequence.

INTRODUCTION

Salmonellae are one of the most important microorganisms that cause disease in man and animals and among the most common causes implicated in outbreaks of food born infectious disease around the world, (Abou-Zeed et al., 2000). Animals are mainly infected through feed, drinking water or environmental sources. 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 (Van, et al., 2006).

Salmonellae have a wide host range, including, human, animals and birds (Douce et al., 1991). Salmonellae cause acute and chronic enteritis, septicemia, abortion, poly-arthritis, nervous manifestation and death (Gorman and Adley 2004).

Salmonellae produce a variety of putative virulence determinants, including adhesins, invasins, fimbriae, exotoxin and endotoxin. (Jones et al., 1982).

Virulence in microorganism is associated with the capacity to attach and colonize at the site of infection, with subsequent damage to the host and is promoted by aggressins that interfere with the host defence (Abou-zeed et al., 2000 and Pasquali et al., 2004).

We have recently cloned a group of genes (invA), that allows *S. Typhimurium* to enter cultured epithelial cells. The invA genes are arranged in the same transcriptional unit. Virulent strains of *S. Typhimurium* carrying defined mutations in invA had higher 50% lethal doses than their parent strains when administered orally to mice and were deficient in their ability to colonize Peyer's patches and the small intestinal wall. In contrast, invA mutants were fully virulent, when administered intraperitoneally, suggesting that the invA genes are only needed for the display of virulence when *S. Typhimurium* is administered by the natural route of entry (Jorge and Roy-Curtiss, 1991). In addition, conduction with transcriptional and translational fusions of reporter genes to invA had established that the expression of the invA genes is regulated by changes in DNA (Swamy et al., 1996).

The isolation and identification of salmonellae by traditional methods is time-consuming and laborious. The polymerase chain reaction (PCR) provides a way of overcoming these difficulties and allows amplification of the target DNA (Murugkar et al., 2003). The purpose of this study was to determine serological and virulence patterns of *Salmonella Typhimurium* isolated from cow calves, lambs and human. All isolates were tested for antimicrobial drug susceptibility. Moreovers, to determine the genotypic characteristics including the presence of virulence invA gene at different isolated.

MATERIAL AND METHODS

Isolation and identification of salmonellae:

A total of 204 faecal samples from cow calves lambs and human were cultured in selenit F broth and incubated at 37°C for 18 hour, a loopful from inoculated broth was streaked onto the surface of S.S. agar plates, then incubated at 37°C for 24 hours. Suspected colonies were identified culturally and biochemically according to and serologically by slide agglutination test using Salmonella Typhimurim antisera.

Detection of virulence factors:

1. Congo red (C.R.) binding activity (Agenta al 1997):

Salmonella strains were cultured onto Congo medium. CR-positive Salmonsella isol were identified by the appearance of red c nies. The reaction was best seen after 24 h cubation at 37°C, followed by an addition

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days at room temperature. CR-negative Salmonella colonies did not bind the dye (white colonies).

2. **Detection of haemolysin (Tang et al., 1993):**

a. B-hemolysin:

Salmonella isolates were inoculated into blood agar plates containing unwashed sheep blood 5%, after 24 hours of incubation at 37°C positive B-haemolysin production was indicated by clear zone of haemolysis.

3. **Adherence assay (Douce et al 1991):**

10 µl of overnight bacterial cultures in peptone water (containing 1% D-mannose) were inoculated into cover slips 24 well plate, which had been seeded with 5 X 10^5 HEP-2 cells 48 hr. Cultures were incubated at 37°C for 3 hr. The cells were washed. Fresh Hanks 199 was added and then the cells were incubated for further 3 hr. Then cells were fixed with 3% formalin and stained with Geimsa solution. The adhesion was determined by light microscopy covering the whole slide. Bacteria were recorded as adhesive if a cluster of at least 10 bacteria adhered per HEP-2cell.

4. **Invasion assays (Dinjus et al., 1998):**

10µl of bacterial culture in peptone water were incubated with HEP-2 cells for 2-3hr to allow attachement and penetration of epithelial cells. Gentamicin (which is unable to penetrate mammalian cells) was added to eliminate extracellular bacteria. The cell sheet was washed, fixed and stained by Giemsa.

In vitro susceptibility of *Salmonella* isolates to various antimicrobial agents:

Antimicrobial drug sensitivities were determined for each Salmonella isolate using disc diffusion method and commercial dises (Djukeren et al., 2003).

Extraction of DNA from bacteria:

The strains were routinely grown for 24 hours at 37°C in 5ml of Luria broth medium in Loose-top culture tubes with aeration at 90 rpm in shaker incubator. The organisms were pelleted by centrifugation at 8000 X g for 10 minutes, washed twice with 10ml of phosphate buffer saline and reuspended in 2ml TE buffer (10mM Tris, 1mM ethylene-diamine tetra-acetic acid (EDTA. pH 7.6). The bacteria were lysed with sodium dodecyl sulphate at the final concentration of 1% and were incubated at 37°C for 1 hour. The mixture was then treated with proteinase K (20mg/ml) and incubated at 37°C for 1 hour. The chromosomal DNA was then extracted twice with an equal volume of phenol: chloroform: isoamyl alcohol (25 : 24 : 1). The DNA was precipitated with 1/10 volume of 3M sodium acetate and 2 volumes of absolute ethanol and incubated overnight at-20°C. the precipitated DNA was pelleted by centrifugation at 8000 Xg at 4°C for 10 minutes and resuspended in 500µl TE buffer. (Amin and mazhar, 1997).

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Oligonucleotide primers:

Two pairs of oligonucleotide primers specific for salmonella invA gene were used for the PCR methods. This primer was predicted to yield a 521 bp product (Jorge and curtiss 1991).

The oligonucleotide primers specific for salmonella invA gene:

The invA sense primer: 5`-TTG TTA CGG CTA TTT TGA CCA-3`

The invA antisense primer: 5` CTG ACT GCT ACC TTG CTG ATG-3`

DNA amplification:

DNA amplification was performed according to Abou-zeed et al. (2000) in a volume 50µl containg 10 mM Tris Hcl pH 8.3, 50 mM Kcl, 1.5 mM $MgCl_2$, 50 ng template DNA, 200µM of each of four deocyribonucleoside triphosphates dATP, dGTP, dCTP and dTTP, 20 PM of each oligonucleotide primers, and 1.5 M of Taq-DNA polymerase enzyme. Samples were subjected to heating for 5 minutes at 94°C then using 35 cycles of denaturation at 93°C for 1 minute, annealing for 1 minute at 52°C and extension for 2 minutes at 72°C. the last cycle was followed by a final 10 minutes extension at 72°C.

Electrophoretic detection of PCR products:

The PCR products were visualized by gel electrophoresis. Samples (10µl) of final PCR products were mixed with loading dye and loaded onto a 1.5% agarose gel and subjected to electrophoresis for 1 hour at 100 V. in LX TBE buffer (Tris Hcl, Boric acid, EDTA) Murugkar et al., (2003).

RESULTS

The result showed that the incidence of Salmonella Typhimurium was (4.76%) and (6.45%) in apparently healthy calves and lambs while in diarrhaeic calves, lambs and human it was (16.07%), (15.15) and (9.52) respectively.

The typing of the tested strains isolated from diarrhoeic calves, lamb. and human as a confirmatory step is shown in Table (2); all examined isolates recovered from diarrheic and apparently healthy animals and human were bounded with Congo red dye giving red colonies 18, isolates (81.81%) and for B hemolysin producer 17 isolates (71.27%).

Results in Table (3) revealed that 15 (68.18%) of Salmonella isolates could adhere to HEP-2 cells and invasion 13 isolates invaded the cells. (59.09%). Results illustrated in Table (4) revealed. The in vitro sensitivity of Salmonella isolates against 10 antimicrobial agents. The examined isolates were proven to be sensitive to Ofloxacin, Gentamicin, Ciprocin, Nitrofurantoin and Chlormphenicol while Ciprocin, Gentamicin and Ofloxacin complete sensitive inhuman.

The optimal reaction condition for amplifying a template DNA was optimized in relation to different factors such as: primer structure, magnesium ion concentration, annealing temperature and DNA polymerase enzyme. The effect of these factors was qualitatively evaluated by determination of the PCR amplification products fractionat-

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ed on agarose gel and visualized under U.V. light after staining with ethidium bromide. The obtained data indicated that the optimal concentration of magnesium ion in the reaction was 1.5μM, Taq polymerase enzyme concentration was 1.5μ, primer concentration was 10 PM and optimal annealing temperature was 52°C. six isolates were examined by PCR to determine the virulence invA gene. All isolates were positive for invA gene sequences as indicated by the size of the PCR product in agarose gels (approximately 521 bp) (Fig. 2).

Table (1): Incidence of salmonella typhimurium isolated from animals and human

Specis	Total number of examined samples	No. of isolated *Salmonella* Typhimurium from animal and human	Apparently Normal		Diarrhoeic animals and human	
			No. of feaoal samples	No. of positive samples	No. of diarrheic fecal samples	No. of positive samples
cow calves	98	11(11.22 %)	42	2(4.76)	56	9(16.07)
Lambs	64	7 (10.93%)	31	2(6.46)	33	5(15.15)
Human	42	4 (9.52%)	-	-	42	4(9.52)
Total	204	22 (10.78%)	73	4(5.47)	131	18(13.74%)

Table (2) Biological characteristics *Salmonella Typhimunium* strains isolated from cow calves, sheep and human.

Source of samples	No. of isolates	Congo red assay		B-haemolysin	
		No.	%	No.	%
Cow calves	11	9	81.8	9	81.8
Lambs	7	5	71.42	5	71.42
Human	4	4	100%	3	75
Total	22	18	81.81	17	77.27

Table (3): Adherence and invasion properties of different *Salmonelle* isolated from cow calves, sheep and human.

Serogroup	No. of isolates	Adherence assay		Invasion assay	
		Positive		Positive	
		No.	%	No.	%
Cow calves	11	8	72.72	7	63.63
Lambs	7	5	71.42	4	57.14
Human	4	2	50	2	50
Total	22	15	68.18	13	59.09

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Table (4): Antimicrobial susceptibility pattern of Salmonella isolates from animal.

Antimicrobial agents	Conc. (μg)	Antimicrobial susceptibility		
		Sensitive	Intermediate	Resistant
Ampicillin	40μg/ml	9/18 (50%)	2/18 (11.11%)	7/18 (38.88%)
Chlormphenicol	30μg	15/18 (83.33%)	1/18 (5.55%)	2/18 (11.11%)
Ciprocin	5 μg	16/18 (88.88%)	-	2/18 (11.11%)
Erythromycin	15 μg	2/18 (11.11%)	1/18 (5.55%)	15/18 (83.33%)
Gentamicin	10 μg	17/18 (94.44%)	-	1/18 (5.55%)
Nalidixic acid	30 μg	10/18 (55.55%)	3/18 (16.66%)	5/18 (27.77%)
Nitrofurantoin	300 μg	16/18 (88.88%)	-	2/18 (11.11%)
Ofloxacin	5 μg	18/18 (100%)	-	-
Streptomycin	10 μg	9/18 (50%)	5/18 (27.27%)	4/18 (22.22%)
Tetracycline	30 μg	1/18 (5.55%)	3/18 (16.66%)	14/18 (77.77%)

Table (5) : Antimicrobial susceptibility pattern of Salmonella isolates from human.

Antimicrobial agents	Conc. (μg)	Antimicrobial susceptibility		
		Sensitive	Intermediate	Resistant
Ampicillin	40μg/ml	3/4 (75%)	-	1/4 (25%)
Chlormphenicol	30μg	1/4 (25%)	1/4 (25%)	2/4 (50%)
Ciprocin	5 μg	4/4 (100%)	-	-
Erythromycin	15 μg	-	1/4 (25%)	3/4 (75%)
Gentamicin	10 μg	4/4 (100%)	-	-
Nalidixic acid	30 μg	-	2/4 (50%)	2/4 (50%)
Nitrofurantoin	300 μg	-	1/4 (25%)	3/4 (75%)
Ofloxacin	5 μg	4/4 (100%)	-	-
Streptomycin	10 μg	-	-	4/4 (100%)
Tetracycline	30 μg	-	-	4/4 (100%)

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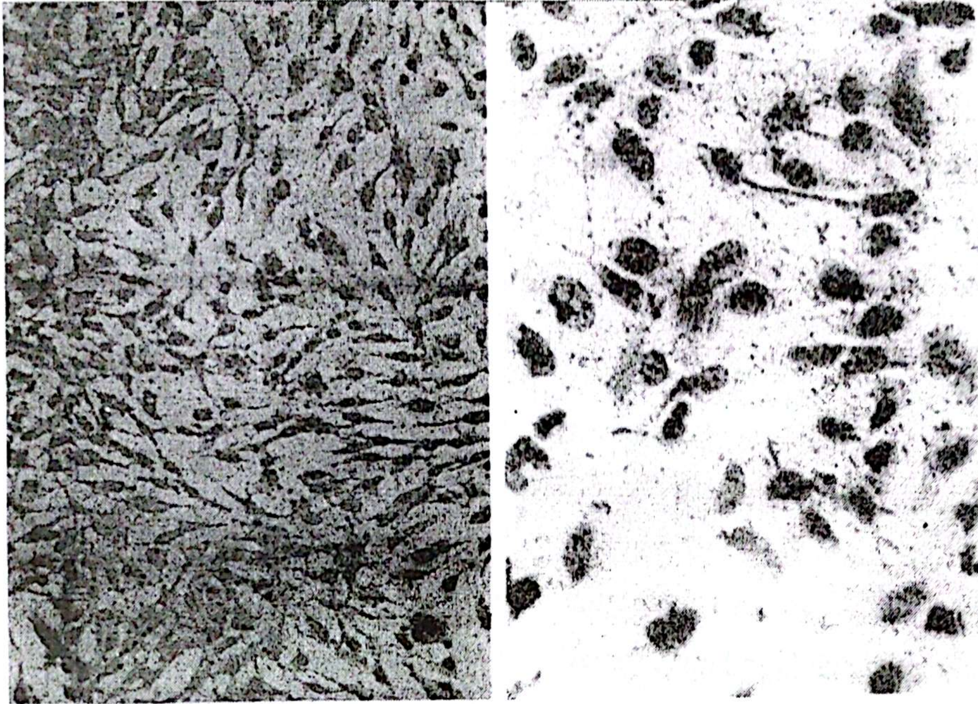

Fig. (1): HEp-2 cell invasion. A= Control negative. B= positive invasion strain.

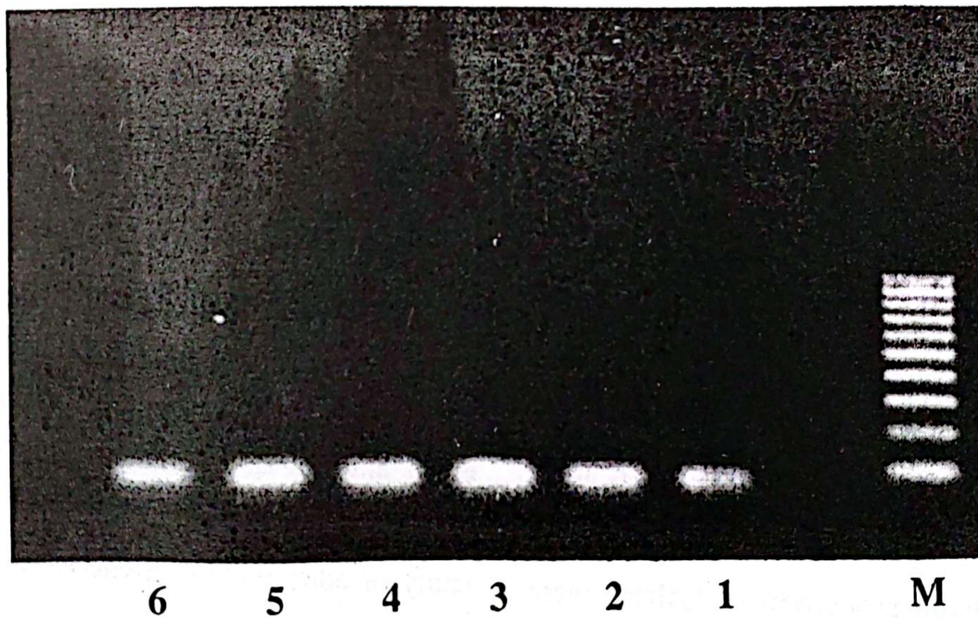

Fig. (2): Analysis of PCR products of invA gene of salmonella isolates of 15% agarose gel electrophoresis Lane M. 100bp ladder; Lane 1 and 2 *Salmonella enterica serovar* Typhimurium Isolates for cow calves. Lane 3 and 4 *Salmonella enterica serovar* Typhimurium Isolates from lambs. Lane 5 and 6 *Salmonella* enterica serovar Typhimurium Isolates from human.

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DISCUSSION

Salmonellae are thought to be the major pathogenes leading to serious economic losses in animal, industry and human health. The highest incidence of Samonella Typhimurium was (11.22% recovered from calves, followed by lambs and human (10.93%) and (9.52%). It is worthly to denote, that our result *S. Typhimurium* agreed with that reported in human (Obil et al., 1997) and calves and lambs (Hemmat zadeh and Khalil , 1988) also these result agree with who reported such the incidency of Salmonella in lambs varied from 0-upto 45%.

Identification of virulence determinants of salmonellae isolates had led to better understanding of pathogenesis of diarrheal disease caused by them and providing a new dimension to their diagnosis. The occurrence of distinct pathogroups could be shown within one serogroup (Gorman and Aldey, 2004). The results illustrated in table (1) showed that all isolates recovered from animals and human bind Congo red. Pathogenic Salmonella have evolved some unique cellular products associated with virulence of organism. The results indicated that a large proportion of isolates were haemolysin producer and this could be used as a phenotypic marker or virulence factor. Even though strains which elaborated haemolysin were frequently associated with diarrhoea and played critical role in extra-intestinal infection, there was no evidence to suggest that the elaboration of haemolysin increases the potential for causing diarrhoea. In addition, its is important to note that the addherance and invasion of micro-organism to HEP-cells is controlled with the invasion of the gastrointestinal mucosa of the organisms which is an critical step in pathogensis caused of Salmonella microorganism (Murugkar et al., 2003).

The ability to adhere to intestinal epithelial cells was an important virulence factor. Adhesion would allow organisms to overcome the disadvantages of living in constantly moving environment. The epithelial surface was likely to provide a more stable environment than the lumen of intestine and the bacteria would be in close approximately to nutrient transport. The results illustrated in table (3) agree with (Agenta et al., 1997), who recorded adhesion at the ranges of 66.77 and 88.1% among *Salmonella* recovered from human and calf. In vitro invasion test revealed that 13 (59.09%) isolates were positive. Khalil (1988) recorded that invasion was at ranges of (36.4%-50%) according to sources of isolates. However, no relationship could be found between adherence, and invasivness. The invasion of HEp-2 cells by salmonella serovars is a useful model for study of adhesive and invasive properties of this pathogen. The penetration of HEp-2 cells appears to result from endocytosis of the bacteria by the animal cells (Jones et al., 1982).

All tested serovears were highly sensitive of *Salmonella* to 10 antimicrobial agents. The

examined isolates were proved to be highly sensitive to ofloxacin, Gentamicin, Ciprocin, Nitrofurantoin and Chlormphenicol for animals isolates while Ciprocin, Gentamicin, Ofloxacin complete sensitive to human isolates (Wedel et al., 2005).

The PCR is a highly accurate method which makes it possible to detect nucleic acid amplification products. The results can be obtained rapidly so that they can be used not only to support bacteriological investigation but also to make the result more reliable (Galan and Curtiss 1991).

In the present study, a PCR was used to amplify samonella-specific target DNA sequences. A new set of oligonucleotides primer taken from the invA gene was used for amplification to detect and identify salmonella serovares. The primers are different from those suggested in previous studies of (Baumler et al., 1997). The difference consisted in the sequence and in the annealing sites on the nucleotide sequence of the invA gene.

The most suitable condition for DNA amplification was magnesium ion concentration, DNA polymerase enzyme concentration and annealing temperature. These agreed with Tanaka et al., (2004), who mentioned that the optimal PCR condition for amplifying a template DNA can vary from one primer to another and necessary to be determined empirically.

The investigation using PCR for the presence of invA gene in this study demonstrated its presence in all examined salmonella isolates irrespective of the serovar or source. This finding was consistent with previous reports (Galan and Curtiss, 1991) that established the presence of invA gene in nearly all salmonella irrespective of serovar or source. The invA gene is important in the invasion of phagocytic epithelial cells and entry into the intestinal mucosa. This was confirmed by Tanaka et al., 2004 who showed that S. typhimurium strains carrying mutation in invA genes are unable to selectively invade in the follicle-associated epithelium of murin Peyer' patches.the results of this study agreed with Murugkar et al., (2003) who reported that all salmonellae possessed the genetic information on invasion (invA), they adhered equally well to epithelial cells, could penetrate into these and survive and multiply inside cells. Since adhesion, invasion and the ability of intracellular survival and multiplication,constitute the most virulence parameter of salmonella.

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