RESULTS

In our study, patients haemoglobin concentration (Hb) ranged from (9-14 g/dl) with the mean was (11.2%), white blood cells ranged from (3.00 - 10.0 x 10^9/l) and the mean was (4.2%).

In this study, it was found that, 30 cases out of 35 studied patients gave positive culture results: 11 cases of them (31.4%) were positive blood culture mostly of them during first week of illness, 17 cases (48.5%) gave positive stool culture mostly during second week, and 2 cases (5.71%) only showed positive urine culture during second week onward. After culture, the growth was confirmed by biochemical reactions and serotyping

The results of Widal test showed high titre.

Bronchoalveolar lavage (BAL) of the cases with chest mainfestation showed negative growth on culture.

- DNA preparation of 30 positive isolate Salmonella typhi showed:

The quality DNA preparation :-

The DNA preparation method yielded high molecular weight DNA whose size distribution was intact (Figure 3). The size distribution of the genomic DNA did not change upon extended incubations at 37°C, indicating the absence of any DNA-degrading enzymatic activity in the

preparation (Figure 4). The purity of the DNA preparation was checked by measuring its optical densities at 260nm (OD260) and at 280 nm (OD 280). The ratio between OD_{260} and OD_{280} ranged from 1.7 to 1.9 (Table 5) indicating that salting-out of proteins by adding a saturated sodium chloride solution satisfactorily deproteinized the DNA preparation, and that the purity of DNA was adequate for enzymatic manipulations and polymerase chain reactions (PCRs).

Oligonucleotide Strategy :

The nucleotide sequence of the IS200 insertion element was analyzed by the oligonucleotide analysis software, Oligo 4 from National Biosyntheses (USA) in order to select specific oligonucleotide primers for the inverse PCR strategy. A plus sense primer 5' TCGGTATTTGGGCGCGAAAA 3' (nucleotide position 493 to 512) and a minus sense primer 5' CTGTTAGGGCGCGGCTGGTA 3' (nucleotide position 73 to 54) were selected for PCR (Figure 1). The dissociation temperatures (Td) of the IS200-specific oligonucleotide primers used in inverse PCR are given in table (4). The plus-sense primer had the following base composition: A+T = 10 (50.0%) and C+G = 10 (50.0%). The dissociation temperature of the plus-sense primer (Td) was 72.6°C as calculated by the nearest neighbor method. The minus-sense primer had the following base composition: A + T = 7 (35.0%) and C+G = 13 (65.0%). The dissociation temperature of the minus-sense primer (Td) was 72.4°C as calculated by the nearest neighbor method. The difference

in the dissociation of the two primers was 0.2°C. Primer secondary structure formation or primer-primer interactions were energetically negligible (Figure 5).

The Specificity of inverse PCR

The genomic DNAs were digested using restriction enzyme PstI that had no recognition site within the IS200 DNA sequence (Figure 1). The restriction digestion products were circularized by ligation using bacteriophage T4 DNA ligase. The circular DNA molecules were used as templates in PCR with IS200-specific oligonucleotide primers as described under subjects and methods and illustrated in Figure (2).

The specificity of the inverse PCR strategy using the IS200-specific oligonucleotide primers was confirmed by the ligation-dependent formation of circular DNA molecules. PCR with plus-and minus-sense primers resulted in the formation of linear DNA molecules constituting the IS200 fingerprint. The PCR products, IS200 bands, were amplified only when the DNA templates were digested by PstI restriction endonuclease and ligated with T4 DNA ligase before PCR (Figure 6). These IS200 bands should correspond to insertion sites carried on PstI fragments. All PCR amplification failed when the DNA templates were digested with PstI restriction endonuclease but not ligated with T4 DNA ligase or ligated with T4 DNA ligase but not digested with PstI restriction endonuclease before PCR.

The IS200 Copy Numbers :

The IS200 mobile element was polymorphic among the studied S. typhi isolates. All 30 S. typhi isolates tested were found to possess at least eight copies of the IS200 element distributed in the bacterial genome on fragments in the range of 0.3 - 3 kbp in size (Table 5 and Figure 7). The location of an IS200 copy corresponding to a constant IS200 band of 0.6 kbp was conserved throughout all S. typhi isolates tested. Thirteen isolates were endowed with a relatively higher IS200 copy number, ranging from 11 to 14 copies per bacterial genome. Seventeen isolates were endowed with a relatively lower degree of IS200 polymorphism, ranging from 8 to 10 copies per bacterial genome (Table 5).

S. typhi Clonal Lines:

It was possible to distinguish 14 clonal lines (CL) among the 30 S. typhi isolates studied. (Figure 7) shows Southern blot of the IS200 fingerprints. The IS200 fingerprint was unique for a given clonal line. and the level of diversity observed among DNA fingerprints of the different S. typhi clonal lines was considerable. The number of isolates and the percentage of occurrence for each clonal line are shown in (Table 6). Among the 30 S. typhi isolates studied, clonal line 5 was the most frequent (20%) line followed by clonal line 1 (13.3%).

S. typhi IS200 Patterns :

Cluster analysis identified four IS200 fingerprinting patterns (PTA) among the 14 S. typhi clonal lines (CL) observed in this study. These

IS200PATs were provisionally designated IS200PAT1, IS200PAT2, IS200PAT3, and IS200PAT4 (Figures 8 and 9). The frequency of occurrence of the different IS200PATs are given in (Table 7). The IS200PAT1 was the most frequent (46.6%) pattern while IS200PAT3 and IS200PAT4 were the least frequent patterns (10% each).

The IS200PAT1 was characterized by IS200 bands of 0.4 kbp, 0.6 kbp, and 0.7 kbp and one or more of the followings: 0.8 kbp 0.9 kbp and higher (Figure 8). IS200PAT2 had DNA bands of 0.4 kbp, 0.6 kbp and 0.8 kbp and one or more of the followings : 0.3 kbp, 1 kbp and 2 kbp. IS200PAT3 had hands of 0.4 kbp, and 0.6 kbp and one or more of the followings : 0.8 kbp and higher.IS200PAT4 had conserved bands of 0.6 kbp, and 0.8 kbp and one or more of the followings: 1 kbp, 2kbp and higher (Figure 8).

Figure (9) shows that the clonal lines were clustered into the different IS200PATs as follows: Six clonal lines encompassing 14 S. typhi isolates were clustered into IS200PAT1 with a high degree of similarity. Four clonal lines encompassing 10 S. typhi isolates were clustered into IS200PAT2. Two clonal lines containing 3 S. typhi isolated were clustered into IS200PAT3. Finally, two clonal lines containing 3 S. typhi isolates were clustered into lS200PAT4

The degree of IS200 polymorphism (copy number) among IS200PATs from 1 to 4 given in (table 8). The IS200 polymorphism ranged from 9 to 14 copies in the bacterial genomes in IS200PAT1, 8 copies in the bacterial genomes in IS200PAT2,9 copies in the bacterial genomes in IS200PAT3, and 8 copies in the bacterial genomes in IS200PAT4. The average IS200 copy number ranges was 11.3 copies in the bacterial genomes in IS200PAT1, 8 copies in the bacterial genomes in IS200PAT2 and IS200PAT4, and 9 copies in the bacterial genomes in IS200PAT3.

TAAATATCCT CCGGCATAGC CGAGGTTTTT CAGATGCGCC TATAACGCTC TCTTACCAGC 60
CGCGCCCTAA CAGGCGCATA CGATCTGACA TTTGCATCAA ACTTCGTTAC TTACGGCCCG 120
TAAATCGGGC TGCCCGGATA GGGGATGGAT AATTGCTCAC CCATTTTATC CTCTTCAAGC 180
TGGIGCTTTA TGTAGTCCTG TATCTTCGCC GTGTTCTTAC CCACCGTATC GACTAGTAC 240
CCTCTGCACC AGAACTCCCT GTTCCTGTAT TTGAATTTTA GATCCCCAAA CTGCTCGTAA 300
AGCATCAGAC TACTTTTACC CTTCAGATAT CCCATGAAAC TCGACACACT CATCTTCGGC 360
GGGATCTTCC AGAAGCTGTG AATATGATCT GCACAACATT DTGCTTCCAG GAAATTCGTAC 420
GTTTTTCCAT TCACACAATT TTCTTAATAT GCTGCCTACT GCCTACGCT TCTCTCCATA 480
GAACGCTTGT CTTCGGTATT TGGGCGCGAA AACTATGTGA TATTTACAGT TCCATCGGGT 540
GTGCGCTAAG CTCTTTTCGT CCCCCATTGG ACCCCCTTTT GATTTCTTGT GGAACTTTTG 600
CAGTTGCCAG ACCGCAAGAT GTTTTAACAA ATCAAAAGGG GTTTTAATAA CTGGCTTAAA 660
GCTGAAAGCT TTCCGGAACC CCCAGCCTAG CTGGGGGTTT CCATAGAC 708

Figure (1) : Nucleotide sequence of the IS200 insertion sequence. Minus sense (73 → 54) and plus sense (493 → 512) primers used in PCR are underlined.

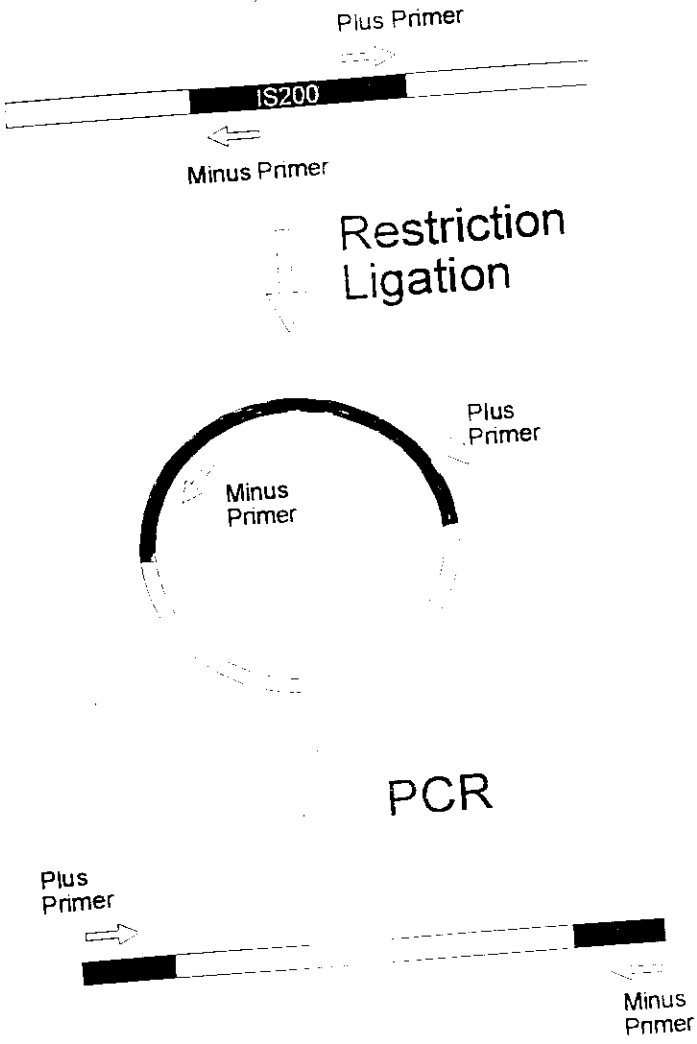


Figure (2): Inverse PCR strategy for IS200 fingerprinting. Genomic DNA is digested with a restriction enzyme that has no cleavage site inside the IS200 element and ligated to form circular DNA molecules. PCR with plus-and minus-sense primers results in the formation of linear DNA molecules.

Sequence of the IS200 is shwon in black while flanking DNA sequences amplified by inverse PCR are shown in white

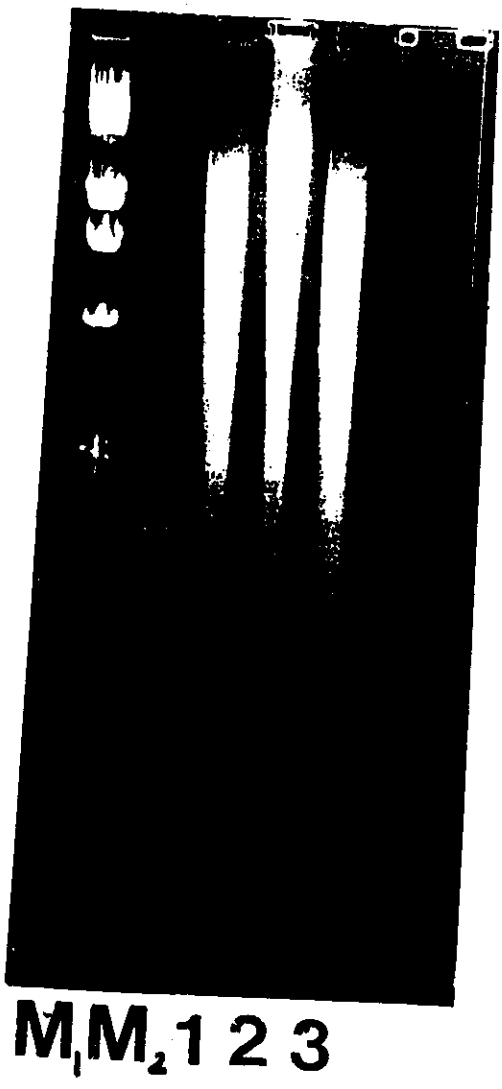


Figure (3): High molecular wight genomic DNA electrophoresed onto a 0.8% agarose gel and stained with ethidium bromide as described under Subjects and Methods.

M1 : High molecular weight marker.

M2 : Low molecular weight marker.

1,2,3: Samples of bacterial DNA.

Figure (4) : High molecular weight genomic DNA before (1) and after (2) overnight incubation at 37°C. The DNA was electrophoresed onto a 0.8% agarose gel and stained with ethidium bromide as described under Subjects and Methods.

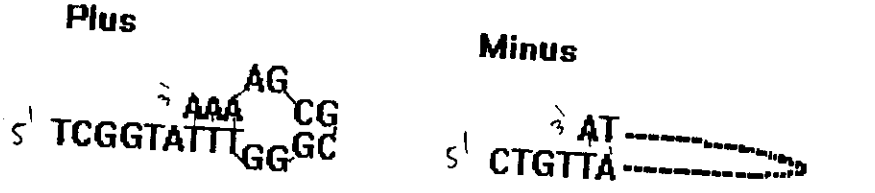


Figure (5): Secondary structures of oligonucleotide primers used for the amplification of IS200-flanking sequences by inverse PCR.

Figure (6): Specificity of inverse PCR strategy. All PCR products were electrophoresed onto a 1.25% agarose gel and stained with ethidium bormide as described under Subjects and Methods.

Lane (1): Non-digested DNA template was ligated before PCR.

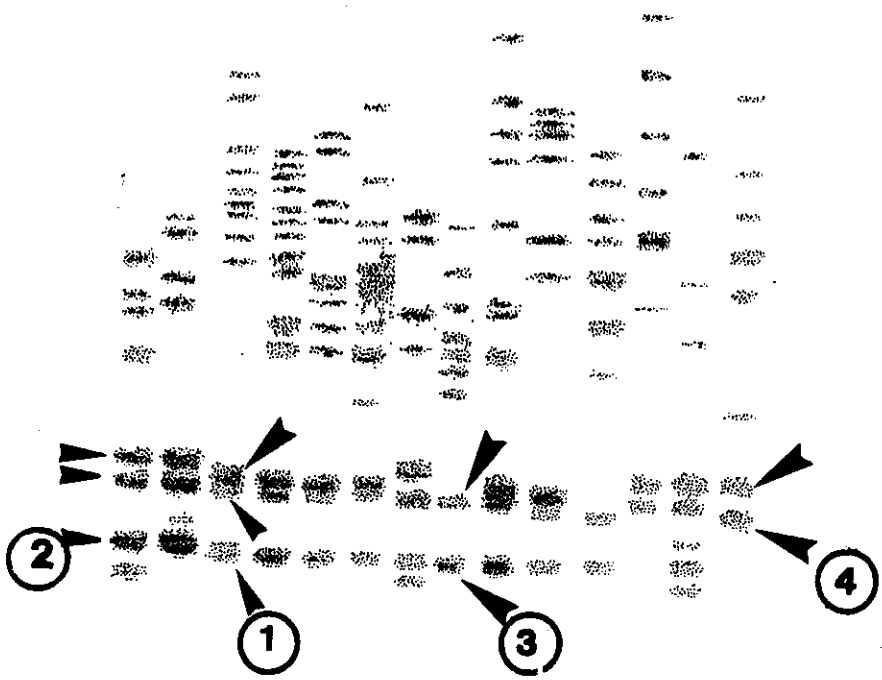
Lane (2): DNA template was digested by PstI but not ligated.

Lane (3): Positive Salmeolla specific PCR product.

Lane (4): DNA template was digested by PstI and ligated with T4 DNA ligase before PCR.

Lane (5): DNA Molecular weight marker.

7



Figrue (7): Southern blot showing IS200 fingerprinting results. All PCR products were electrophoresed onto a 1.25% agarose gel, blotted onto nylon membranes; and processed for chemiluminescence detection as described under Subjects and Methods. Lanes 1 to 14 clonal lines (CL) identified among the 30 S. typhi isolates.

8

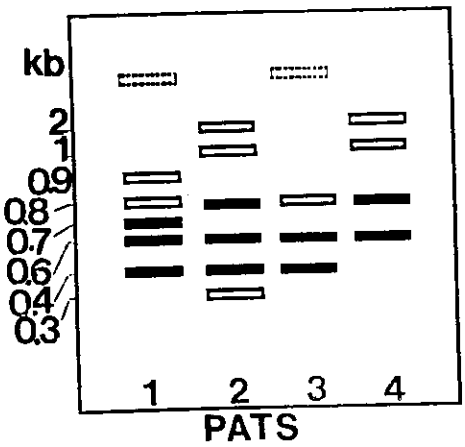


Figure (8): Diagramatic representation of the four IS200 fingerprinting patterns (IS200PAT1 IS200PAT2, IS200PAT3, IS200PAT4) as resolved by electrophoresis onto 1.25% agarose gels.

- PAT-Specific IS200 bands.
- Variable IS200 bands.
- High molecular weight IS200 bands.

9

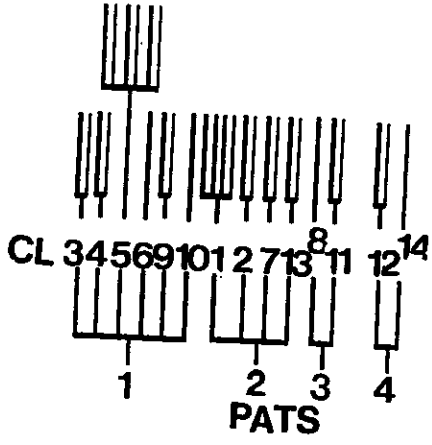


Figure (9): Cluster analysis of the clonal lines identified in the present study. Fourteen IS200 fingerprints were identified but only four patterns designated IS200PAT1 through IS200PAT4 existed.

Table (1) showing positive cultures and Widal test among 35 typhoid

No of patient	Blood culture	Stool culture	Urine culture	Widal test
1	+			+
2	+			
3		+		+
4	+			
5	+			
6	+			+
7		+		+
8		+		+
9		+		+
10			+	+
11		+		
12	+			
13	+			+
14		+		+
15		+		
16	+			+
17		+		+
18		+		+
19		+		+
20			+	+
21		+		+
22				+
23				+
24				+
25		+		
26	+			
27	+			+
28		+		+
29		+		+
30				+
31				
32	+			+
33		+		+
34		+		+
35		+		
Total	11	17	2	25

Table (2) showing positive culture and Widal test during the first, second and third week of typhoid fever.

Test	First week	Second week	Third week	No of patients
Blood culture	9	2		11
Stool culture	4	12	1	17
Urine culture		1	1	2
Widal test		15	10	25

Table (3) showing the percentage of positive cultures among 35 typhoid patients.

Total of positive culture	Blood culture	Stool culture	Urine culture	percentage
11	+ ve			31.4 %
17		+ ve		48.5 %
2			+ ve	5.71 %

Table (4): Nucleotide sequences, positions, dissociation temperature (Td) of the minus and plus sense IS200 - specific oligonucleotide primers used in inverse PCR.

Primer	Sense	Position	Length	Sequence	Td
M1	Minus	73-54	20-mer	5'CTGTTAGGGCGCGGCTGGTA 3'	72.4°C
p2	Plus	493-512	20-mer	5' TCGGTATTTGGGCGCGAAAA 3'	72.6°C

Table (5):

DNA purity and IS200 copy number among isolates tested:

ISOLATE	CLONAL LINE	OD260/280	IS200 COPIES
1	13	1.70	8
2	4	1.82	14
3	9	1.75	11
4	5	1.80	11
5	1	1.79	8
6	7	1.86	8
7	11	1.74	9
8	2	1.70	8
9	5	1.74	11
10	6	1.80	11
11	11	1.74	9
12	4	1.85	14
13	5	1.72	11
14	1	1.74	8
15	8	1.83	9
16	10	1.80	9
17	7	1.73	8
18	3	1.72	12
19	5	1.70	11
20	2	1.74	8
21	9	1.80	11
22	14	1.79	8
23	5	1.84	11
24	1	1.89	8
25	12	1.72	8
26	1	1.90	8
27	3	1.72	12
28	13	1.81	8
29	12	1.80	8
30	5	1.72	11

Table (6)

IS200 Patterns (PAT) of S. typhi clonal lines (CL) identified:

Clonal Line #	IS200PAT	Number of Isolates	Percentage
1	2	4	13.3%
2	2	2	6.6%
3	1	2	6.6%
4	1	2	6.6%
5	1	6	20%
6	1	1	3.3%
7	2	2	6.6%
8	3	1	3.3%
9	1	2	6.6%
10	1	1	3.3%
11	3	2	6.6%
12	4	2	6.6%
13	2	2	6.6%
14	4	1	3.3%
14 clonal lines	4 PATs	30 Isolates	100%

Table (7)

Frequency of IS200 patterns (PAT) identified during this study:

	Among clonal lines		Among isolates	
IS200PAT	**Frequency**	**Percentage**	**Frequency**	**Percentage**
1	6	42.85%	14	46.6%
2	4	28.56%	10	33.3%
3	2	14.28%	3	10.0%
4	2	14.28%	3	10.0%
Total	**14**	**100%**	**30**	**100%**

Table (8):

Polymorphism of IS200 among IS200PATs identified during this study:

IS200PAT1		IS200PAT2		IS200PAT3		IS200PAT4	
CL	C.NO	CL	C.NO	CL	C.NO	CL	C.NO
3	12	1	8	8	9	12	8
4	14	2	8	11	9	14	8
5	11	7	8				
6	11	13	8				
9	11						
10	9						
A	11.3	A	8	A	9	A	8
R	9-14	R	8	R	9	R	8

A : average.

CL : clonal line.

C.NO. : copy number.

R : range.