

A STUDY OF SALMONELLA, SHIGELLA AND ENTEROPATHOGENIC ESCHERICHIA COLI SEROTYPES IN ACUTE GASTROENTERITIS CHILDREN UNDER THE AGE OF FIVE

ALI AKBAR VELAYATI, KIUMARS GHAZI SAIDI, MOHAMMAD-REZA TARAVATI

From the Department of Pediatrics and College of Health Sciences, Tehran University of Medical Sciences, Tehran, Islamic Republic of Iran.

ABSTRACT

In this study, it has been our purpose to isolate and serotype enteropathogenic *E. coli*, salmonella and shigella and determine their resistance to antibiotics. 502 swab specimens were collected during mid-July to mid-November, 1986, from two children's hospitals in Tehran. From 502 cultured specimens, 108 specimens were positive for salmonella, shigella and enteropathogenic *E. coli*, corresponding to 21.5% of the cases. The frequency of the isolated species were salmonella, 56 cases (11.1%); enteropathogenic *E. coli*, 47 cases (9.4%), and shigella, 5 cases (1%).

INTRODUCTION

Acute gastroenteritis, diagnosable through such clinical manifestations as diarrhea, sometimes associated with vomiting, fever, prostration and dehydration, is one of the important diseases of children and infants. In areas with lower economic and hygienic levels and a concentrated population, this disease is especially prevalent.

On the basis of the statistics published by WHO, each year throughout the world about 12 million children die from various diseases, 5 million of whom fall victim to diarrhea. That is, each and every minute, 10 children lose their lives because of this readily diagnosable and preventable disease. The majority of mortalities take place in developing countries. Acute diarrhea is presently one of the most important health problems and basic difficulties of our country's health and treatment systems. In Iran, each year about 70 thousand children under the age of 5 die of diarrhea.

As a rule acute diarrheas have an infectious etiology; they are caused by microbial toxins produced in intestinal mucosa or by ingestion of contaminated food or the invasion of the cells of the intestinal wall by bacteria. Factors causing diarrhea vary according to social and economic conditions, contamination of drinking water and food stuff, culture and customs, and

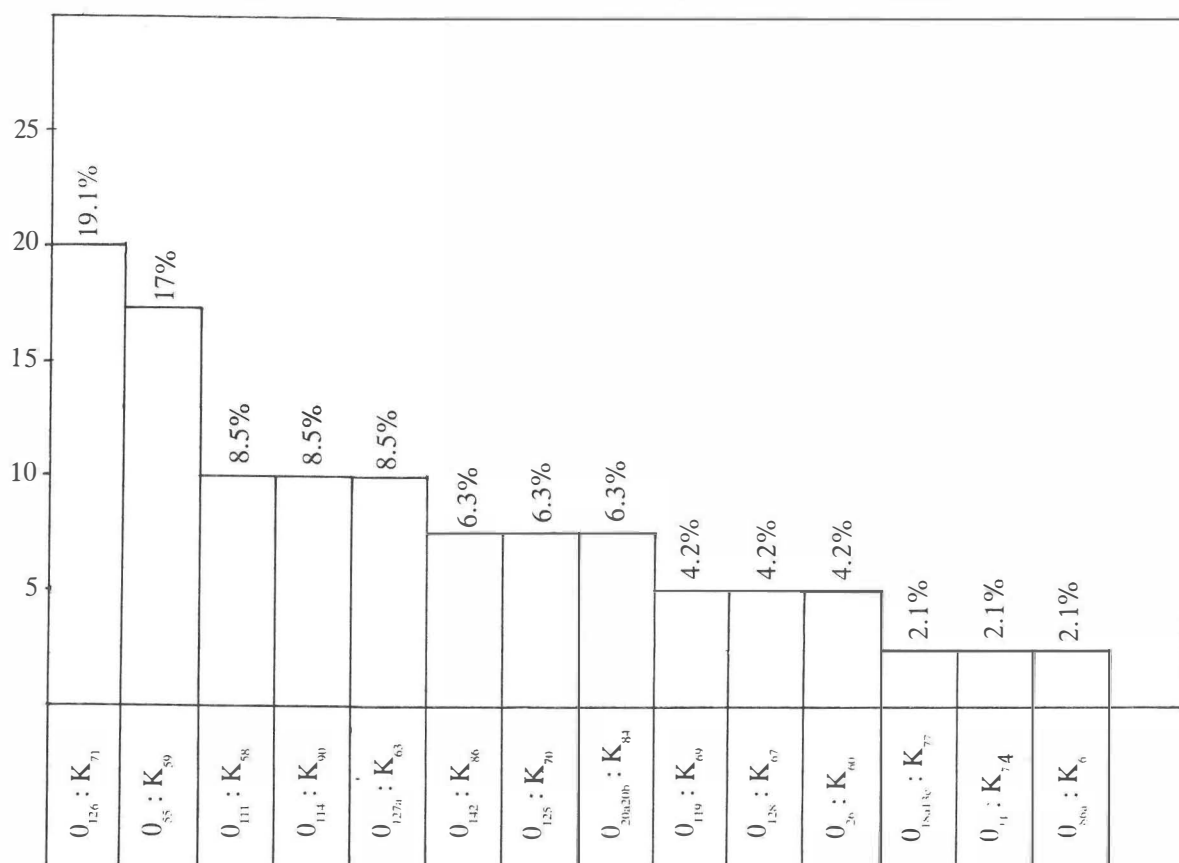
season.^{5,6,7} Besides, the causes of diarrhea vary with the climate. That is to say, in tropical areas, toxicogenic *Escherichia coli* causes diarrhea in the summer, while in areas with a temperate climate, rota-virus causes diarrhea in the winter.

Receptors of intestinal cells, and the acidity and immunological state of the stomach are all among predisposing factors of the host which are determined by lack of protective immunoglobulins present in the intestinal mucosa entering the child's body through maternal milk. Immunosuppressive drugs are also among predisposing factors of diarrhea. It has also been observed that certain surgical operations, e.g. bone marrow graft, when carried out on children, render them susceptible to causative factors of diarrhea. The causes of some types of diarrhea are still unknown.

As far as pathogenic factors are concerned, viruses, bacteria, fungi and parasites are all involved in the causation of endemic and epidemic diarrhea^{8,9}. Extensive studies have been conducted on the etiology of diarrhea in third world and industrialized countries.

In studies recently conducted in the United States, it was discovered that salmonella, shigella and, to some extent, campylobacter, are the main bacteria that cause diarrhea in that country.

In recent years, viruses, particularly rotaviruses,

Histogram No 1: Frequency of Enteropathogenic *E. Coli* Serotypes Isolated at the Children's Medical Center and Takhti Children's Hospital, both in Tehran.

have been known to be the main cause of most of the cases of gastroenteritis in developed countries. A study conducted in the United States, showed that Rotaviruses have been the cause of 19% of cases^{7,10,11}.

METHOD AND MATERIAL

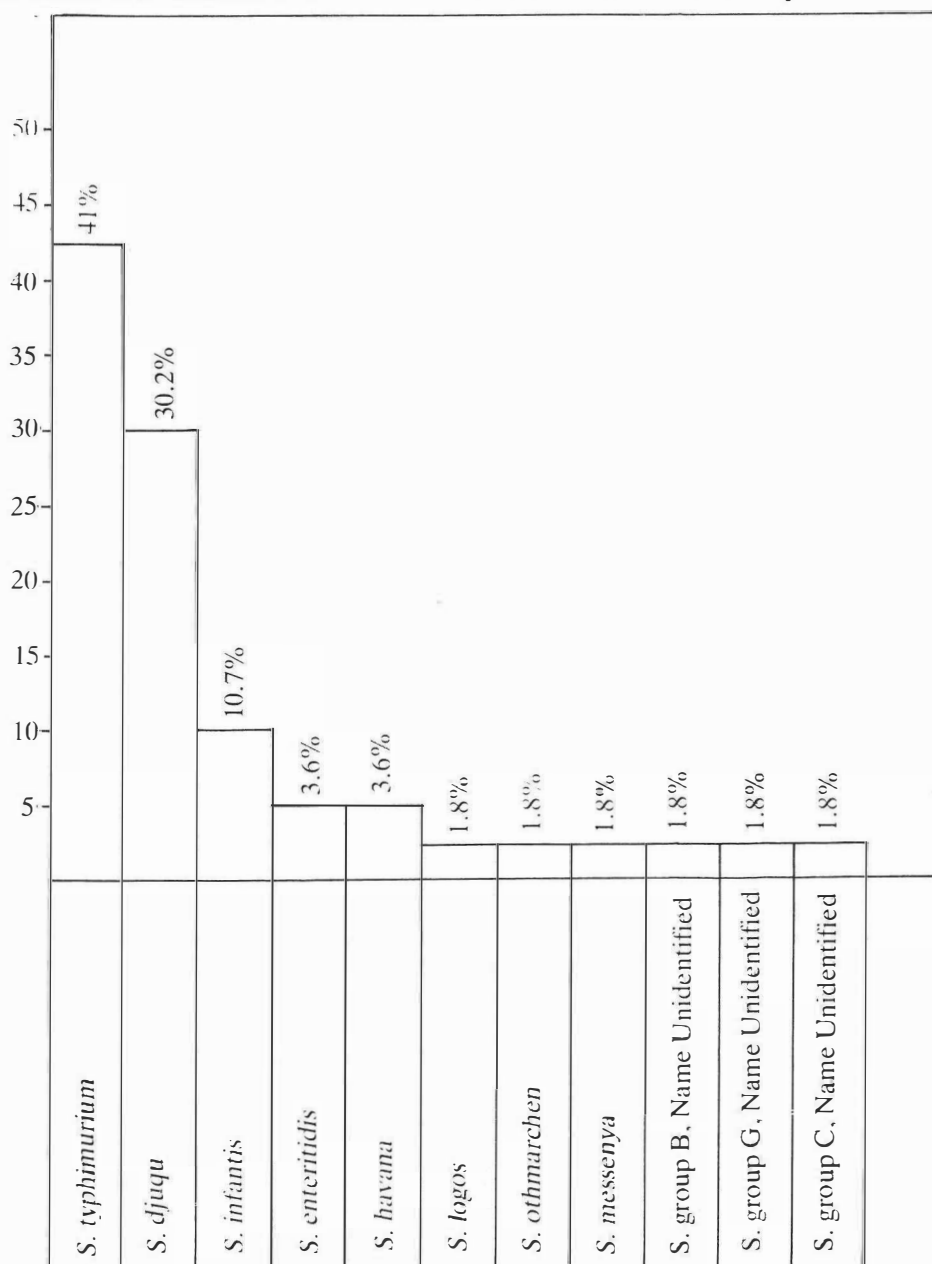
- 1. Patients** During the period from mid-November, 1986 until present, swab specimens from 502 children in the age group 0-5 years were collected in the Children's and Takhti Children's Hospital in Tehran. Out of this total, 200 specimens were taken from the Children's Medical Center, and the other 302 from Takhti Children's Hospital. Most of the subjects were inpatients but some of them were outpatients who referred to these two hospitals' clinics, where specimen collection was carried out.
- 2. Specimen Collection and Transport.** Sterile cotton swabs, soaked in feces, or rectal swabs collected from patients were placed in screw-capped bottles containing Cary-Blair transport medium, labelled with patients, specifications, and transferred to the college laboratory.
- 3. Culturing**
Each fecal specimen was cultured on a blood agar,

deoxicholate agar, Endo's agar and a liquid selenite-F enrichment broth. After 18 hours of incubation at 37°C, the solid media (blood agar, deoxicholate agar, and Endo's agar) were examined. Colorless, black and pale pink colonies were then sub-cultured on differential media (TSI, Kligler iron, SIM, Urea agar, Simmons' citrate agar and decarboxylase).

For further confirmation and certainty of all inoculations an API-20E commercial kit was used. The same day, a sub-culture was made from the liquid selenite-F enrichment broth on salmonella-shigella agar. All inoculations were placed in an autoclave for a 18-24 hours at 37°C, after which the differential media were examined and the biochemical characteristics of the bacteria were determined. Those media that were confirmed to contain salmonella and shigella, were then sub-cultured from TSI medium onto plain gelatin contained in a thin tube especially used for bacteria preservation. After 18 hours of incubation at 37°C, the tubes were sealed by Bunsen burner and preserved at 8°C.

Escherichia coli colonies which had grown on Endo's agar and blood agar were sub-cultured on differential media. Twenty-four hours later when the microorganism was confirmed, a slide agglutination test with

Histogram No 2: Frequency of Paratyphoid Salmonella Isolated from 502 Fecal Specimens of Infants and Newborn Collected at Children's Medical Center and Takhti Children's Hospital, both in Tehran



trivalent, nanovalent, biomeriox antisera, was performed.

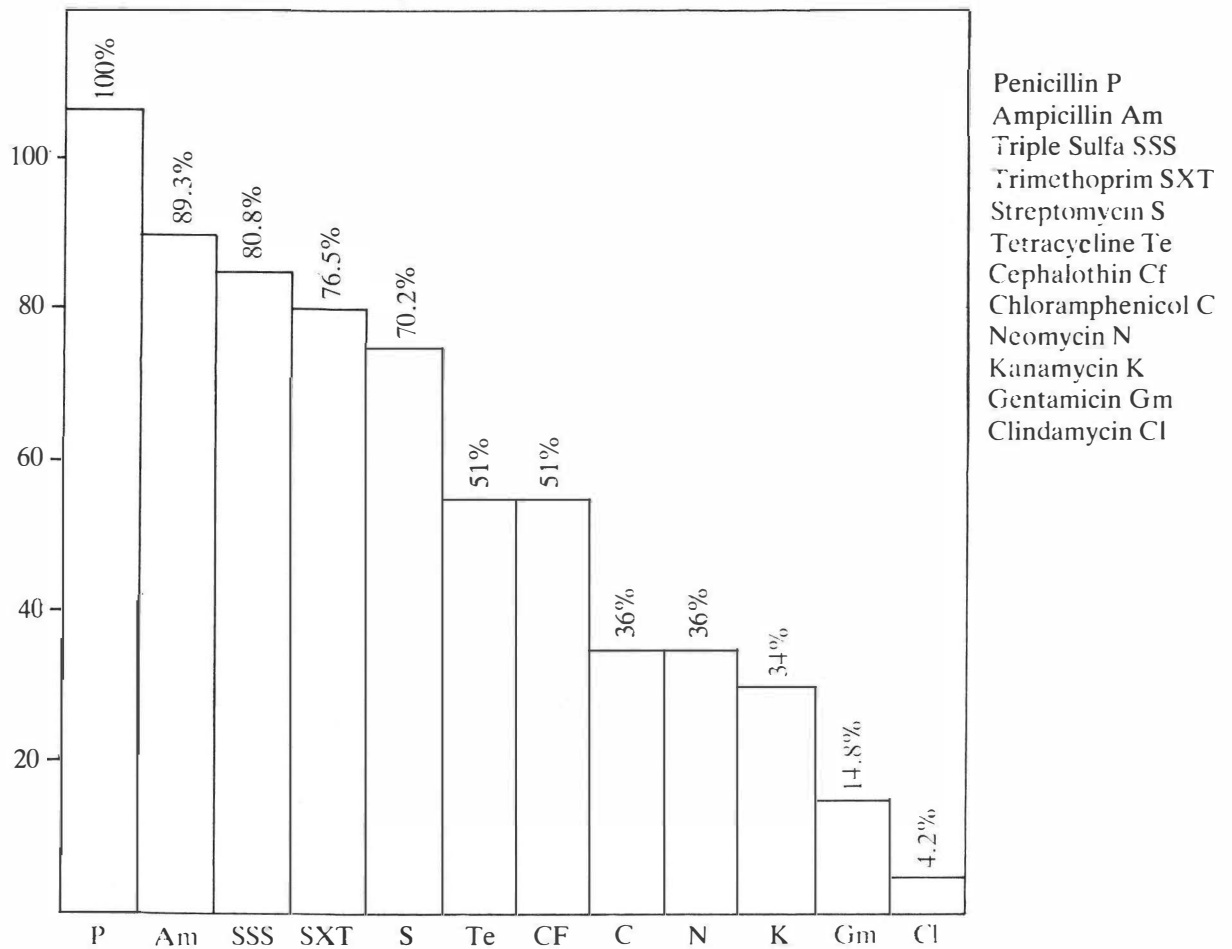
Having confirmed the existence of enteropathogenic *E. Coli*, specimens were collected from 10 positive colonies and sub-cultured in thin tubes according to the above-mentioned procedure. The fact that the bacteria were preserved in thin tubes was due to a lack of specific and monovalent antisera in the college laboratory at the time the specimens were being tested.

Having cultured 502 fecal specimens in the above procedure and prepared the varieties of anti-polyvalent and anti-monovalent *E. Coli*, salmonella

and shigella, the thin tubes were gradually opened and a sub-culture was made on a broth medium. After 6-8 hours in an autoclave at 37°C, the suspected specimens of salmonella and shigella were sub-cultured on salmonella-shigella agar and deoxicholate citrate medium, and the enteropathogenic *E. Coli*, specimens were sub-cultured on blood agar.

After being certain of the specimen's purity, a single colony was transferred by sterile loop, and dissolved in 2cc physiological fluid: with the aid of an API-20E commercial kit, salmonella, shigella and *E. Coli*, microorganisms were confirmed in view of their bioche-

Histogram No 3: Resistance Pattern of Enteropathogenic *Escherichia Coli* Isolated from 502 Cultures of Fecal Specimens Versus Antibiotics and Sulfonamides



mical properties. Following the identification of each of the above microorganisms, necessary measures were taken for the preparation of salmonella, shigella, and enteropathogenic *E. Coli* serotypes.

Sensitivity determination of salmonella, shigella and *E. Coli*, microorganisms towards antibiotics and sulfonamides (disc antibiograms)

Having prepared various salmonella, shigella, and *E. Coli*, serotypes from pure colonies, using the Kirby-Bauer method, sensitivity determinations of the said microorganism were made for 15 antibiotics and sulfonamides¹²⁻¹³.

Five to six colonies previously cultured on plain gelatin medium whose serological and biochemical properties had been studied were sub-cultured in 5 ml trypticase soy broth. The tubes were then placed in an incubator at 37°C for 2-5 hours until the usual turbidity, appropriate to culture developed. At the end of incubation period, the cultures were diluted to the extent that they were proportional to the specifications of standard tubes, as described below:
BaCl₂ 1% 0.5 ml

H₂SO₄ 36% 99.5 ml. In order to determine the sensitivity of the said microorganisms, Muller Hinton agar, 5 millimeters in thickness, was used. Prior to culturing, the media were placed in an incubator for 10 minutes. A sterile cotton swab was then soaked in the microbial solution prepared with a standard dilution; and before sub-culturing, additional liquid was removed from the swab by pressing it against the tube wall. Then, it was evenly spread over the whole surface of the Muller medium. Five minutes later, when the medium surface was dry, the relevant discs were transferred onto the medium and the plates were placed in an incubator at 37°C for 18 hours. The diameter of the nimbus formed around the disc was measured from the posterior surface of the plate, to which the 6 millimeter diameter of the disc was added. Using the Kirby-Bauer standard table, the microorganisms were classified as sensitive, intermediate sensitivity, and resistant, corresponding results were then as follows:

At first, the diameter of the nimbus formed around the disc was measured by a millimeter ruler, from the posterior surface of the plate, to which the 6 millimeter

Table No 1: Frequency of Enteropathogenic *Escherichia Coli* Serotypes, Isolated at Children's Medical Center and Takhti Children's Hospital, both in Tehran

Children's Medical Center				Takhti Children's Hospital		
Serotype	No	%	Serotype	No	%	
0 ₁₂₆ :K ₇₁	4	21	0 ₁₂₆ :K ₇₁	5	17.8	
0 ₁₁₁ :K ₅₈	2	10.5	0 ₁₁₁ :K ₅₈	2	7.1	
0 ₅₅ :K ₅₉	3	15.7	0 ₅₅ :K ₅₉	5	17.8	
0 ₁₁₄ :K ₉₀	-	-	0 ₁₁₄ :K ₉₀	4	14.2	
0 _{127a} :K ₆₃	3	15.7	0 _{127a} :K ₆₃	1	3.5	
0 ₁₄₂ :K ₈₆	-	-	0 ₁₄₂ :K ₈₆	3	10.7	
0 ₁₂₅ :K ₇₀	1	5.1	0 ₁₂₅ :K ₇₀	2	7.1	
0 _{20a20b} :K ₈₄	3	15.7	-	-	-	
0 ₁₁₉ :K ₆₉	-	-	0 ₁₁₉ :K ₆₉	2	7.1	
0 ₁₂₈ :K ₆₇	1	5.2	0 ₁₂₈ :K ₆₇	1	3.5	
0 ₂₆ :K ₆₀	1	5.2	0 ₂₆ :K ₆₀	1	3.5	
0 _{18a18c} :K ₇₇	-	-	0 _{18a18c} :K ₇₇	1	3.5	
0 ₄₄ :K ₇₄	1	5.2	0 ₄₄ :K ₇₄	-	-	
0 _{86a} :K ₆₁	-	-	0 _{86a} :K ₆₁	-	-	
	19	100%		28	100%	

diameter of the disc was added. Using Kirby Bauer standard table, the microorganisms were classified as Sensitive, of intermediate Sensitivity, and Resistant, on the basis of the nimbus diameter.

RESULTS

A group of 502 infants and newborn under the age of five who had contracted diarrhea were examined and made the subject of a study in two hospitals in Tehran. From this number, a total of 108 cases of salmonella, shigella and enteropathogenic *E. Coli*, corresponding to 21.5% of all cases, were isolated.

The above 108 cases comprise 56 strains of salmonella, 47 strains of enteropathogenic *E. Coli*, and 5 strains of shigella. In addition, sensitivity tests towards drugs were carried out on all the isolated pathogenic bacteria, the full result of which are given in the relevant histograms and tables.

DISCUSSION

Acute infectious diarrhea is a prevailing disease everywhere in the world. Annual incidence of this disease in Asia, Africa, and Latin America is much higher than the rest of the world. In Iran, this disease is very common, particularly, in certain seasons, when the number of patients calling on hospitals and clinics is unimarginably high. Fortunately, most factors causing the disease are known; but there are still some unidentified causes for this disease. In these cases, the disease is

attributed to the change of the climate and malnutrition.¹

In Iran, we do not have precise statistics about children mortality caused by acute diarrhea. Nevertheless, in accordance unofficial statistics, each year in Iran about 70,000 children under five years of age die because of this disease. Lack of sanitary facilities and poor living conditions are among the major causes of diarrhea. Studies carried out in India have shown that 58.9% of children suffering from diarrhea caused by salmonellae, shigella, and enteropathogenic *Escherichia coli*, were in the group of below 2 years.¹⁴

On the other hand, research conducted by WHO in various parts of the world have demonstrated that diarrhea caused by salmonella, occurs in the first years and especially the first months of children's life.

Other studies have shown that in developing countries, salmonella spreads by hospital infections and contaminated food. In developed countries however, infection spreads mainly through sewage systems.

A Comparison of the results of the research conducted by WHO and the studies carried out by the Indian Health Institute at various age groups has shown that the results from this institute coincides with those from WHO.

A study on children's diarrhea conducted in the US in 1984 has shown that in a group of 274 children of less than 2 years of age suffering from diarrhea, 62 cases were caused by shigella, 33 by enteropathogenic *Escherichia Coli*, and 5 by salmonella.¹⁵

In Thailand, in a group of 200 children suffering from

Table No2 -Antigenic Schema of Isolated Salmonella, as Demonstrated in This Study

Salmonella Species	group	Sub-group	O antigens (somatic) antigenic formula	H (flagellar) antigens	
				1st phase	2nd phase
<i>S. havana</i>	G	G	1,13,23	t.g.(S)	-
<i>S. typhimurium</i>	B	-	1,4,(5),12	I	1-2
<i>S. logos</i>	B	-	1,4,(5),12	I	1-5
<i>S. infantis</i>	C	C ₁	6,7	r	1-5
<i>S. djugu</i>	C	C ₁	1,9,12	Z=10	c.n.x
<i>S. enteritidis</i>	D	D ₁	1,9,12	g.m	(1-7)
<i>S. othmarchen</i>	C ₁	-	6,7	gm(t)	-
Name Unidentified	B	-	1,4,5,12	I	Undetermined
Name Unidentified	G	G ₂	13,23	Undetermined	Undetermined
Name Unidentified	C	C ₁	6,7	Undetermined	Undetermined

Table No 3: Number and Percentage of Various Species of Salmonella Isolated at Children's Medical Center and Takhti Children's Hospital, both in Tehran

Salmonella Species	Children's Medical Center		Takhti Children's Hospital	
	No	%	No	%
<i>S. havana</i>	6	27.2	17	50
<i>S. typhimurium</i>	7	31.8	10	29.4
<i>S. djugu</i>	4	18.1	2	5.8
<i>S. logos</i>	1	4.5	-	-
<i>S. infantis</i>	2	9	-	-
<i>S. messenya</i>	1	4.5	-	-
<i>S. enteritidis</i>	1	4	1	2.9
<i>S. othmarchen</i>	-	-	1	2.9
<i>S. Group B, Name Unidfd</i>	-	-	1	2.9
<i>S. Group G₂, Name Unidfd</i>	-	-	1	2.9
<i>S. Group C₁, Name Unidfd</i>	-	-	1	2.9
	22	100%	34	100%

diarrhea, 4 cases were proved to be caused by enteropathogenic *Escherichia coli*, 10 by salmonella, 28 by *Shigella flexneri* and 16 by *Shigella boydii*.

Studies conducted in Japan have shown that in this country, invasive *Escherichia coli* is one of the major causes of diarrhea, while in other parts of Asia, with a high incidence of diarrhea, this microorganism has proved to be an insignificant cause of diarrhea.¹⁰

In a research carried out in Nigeria, rectal swab specimens were collected from 84 newborns and children below the age of five. In 10 cases, enteropathogenic *Escherichia coli* and in one case salmonella, were isolated, which indicated that at the time the study was conducted enteropathogenic *Escherichia coli* was the major cause of diarrhea. Another study carried out

in the rural areas showed that in the said area, domestic animals acted as intermediaries for the transfer of bacterial factors. In this area, the isolated cases of salmonella were much less than other microorganisms like campylobacter, which should be attributed to eating habits of the people of that area.¹⁵

The Comparison of the results registered in different parts of Iran with other countries of the world has shown that causes of diarrhea vary with living conditions and in different seasons of the year. Living and economic conditions, contamination of water and foodstuff, culture, customs, and immunological state of the people are among the main causes of the incidence of diarrhea, or the epidemic spread of this disease.

A comparison of the results of all studies carried out

Table No 4: Age Distribution of Patients Suffering from Gastroenteritis Caused by Enteropathogenic *Escherichia Coli* and Paratyphoid Salmonella

Frequency of Diarrhea Caused by Enteropathogenic <i>Escherichia Coli</i> in 47 patients, Expressed in terms of Age			Frequency of Diarrhea Caused by Paratyphoid Salmonella in 56 Patients, Expressed in Terms of Age		
Age (Years)	No	%	Age (Years)	No	%
-1	31	66	-1	42	75
1	7	15.2	1	4	7.1
2	3	6.3	2	5	8.9
3	2	4.3	3	0	0
4+	3	6.3	4+	1	1.7

Table No 5 Study of Resistance Spectrum of Enteropathogenic *Escherichia Coli* Versus Antibiotics and Sulfonamides

Antibiotics Sulfonamides	Abbr.	Resistant		Intermediate Sensitivity		Sensitive	
		%	No	%	No	%	No
Polymixin B	PB	-	-	-	-	100	47
Nalidixic Acid	NA	-	-	6.3	3	93.6	44
Clindamycin	Cl	4.2	2	2.1	1	93.6	44
Furadantin	Fd	-	-	14.8	7	85	40
Gentamicin	Gm	14.8	7	-	-	85	40
Chloramphenicol	C	36	17	-	-	63.8	30
Kanamycin	K	3	16	2.1	1	63.8	30
Neomycin	N	36	17	2.1	1	61.7	29
Tetracycline	Te	51	24	2.1	1	46.8	22
Cephalothin	Cf	51	24	19.1	9	29.7	14
Trimethoprim	SXT	76.5	36	-	-	23.4	11
Triple Sulfa	SSS	80.8	38	-	-	19.1	9
Streptomycin	S	70.2	33	14.8	7	14.8	7
Ampicillin	Am	89.3	42	4.2	2	6.3	3
Penicillin	P	100	47	-	-	-	-

in various parts of Iran shows that infections caused by salmonella are increasing and gradually replacing cases caused by Shigella.

Apart from *Salmonella havana* and *Salmonella typhimurium*, other paratyphoid species, e.g., *S. djugu*, *S. logos*, *S. othmarchen*, *S. messenya*, *S. infantis* and *S. enteritidis*, have also been found. Except for *S. infantis* and *S. enteritidis*, the other four salmonellae species, rarely observed in Iran, appear to belong to new colonies.

One of the prominent points in this study is the inseparability of *S. typhi* species (e.g. *Salmonella typhi*) from *S. Paratyphi* species.

Enteropathogenic *Escherichia coli* Serotypes

The relationship between various serotypes of enteropathogenic *Escherichia coli* and intestinal infections of children below the age of 3, has been known for over 35 years.^{5,16} Further studies have better demonstrated this relationship.^{14,16,17,18} In each region, a special serotype is considered as the cause of diarrhea.^{14,16,17,18,19} A comparison of the results of studies conducted in Iran and in other parts of the world demonstrate that in Iran diarrhea is caused mainly by enteropathogenic *Escherichia coli* serotypes 0₁₁₁, 0₁₂₄, 0₁₂₇, 0₁₂₈, 0₁₂₆ and 0₃₅.

Table No6- Study of Antibiotic Resistance Spectrum of Salmonella Isolated from 56 Cases of Diarrhea in Children of 0-5 Years Age Group, at Children's Medical Center and Takhti Children's Hospital, both in Tehran, Summer 1986.

Antibiotics Sulfonamides	Abbr.	Resistant		Intermediate Sensitivity		Sensitive	
		%	No	%	No	%	No
Nalidixic Acid	NA	1.7	1	3.4	2	94.6	53
Polymixin B	PB	3.6	2	1.7	1	94.6	53
Furodantin	Fd	8.9	5	-	-	91	51
Cephalothin	Cf	55.3	31	10.8	6	34	19
Tetracycline	Te	78.5	44	1.7	1	19.6	11
Neomycin	N	82.1	46	1.7	1	16	9
Trimethoprim	SXT	8.6	49	-	-	12.5	7
Chloramphenicol	C	87.6	49	1.7	1	10.7	6
Gentamicin	Gm	89.2	50	1.7	1	8.9	5
Ampicillin	Am	92.8	52	-	-	7.1	4
Triple Sulfa	SSS	94.6	53	-	-	5.3	3
Kanamycin	K	94.6	53	-	-	5.3	3
Streptomycin	S	94.6	53	5.3	3	-	-
Penicillin	P	100	56	-	-	-	-

Table No 7- Study of Antibiotic Resistance Spectrum of Salmonella havana

Antibiotics Sulfonamides	Abbr.	Resistant		Intermediate Sensitivity		Sensitive	
		%	No	%	No	%	No
Nalidixic Acid	NA	-	-	-	-	100	23
Furadantin	Fd	-	-	-	-	100	23
Polymixin B	PB	4.3	1	4.3	1	91.3	21
Neomycin	N	91.3	21	-	-	8.6	2
Tetracycline	Te	95.7	22	-	-	4.3	1
Chloramphenicol	C	95.7	22	-	-	4.3	1
Cephalothin	Cf	95.7	22	-	-	4.3	1
Trimethoprim	SXT	95.7	22	-	-	4.3	1
Gentamicin	Gm	95.7	22	-	-	4.3	1
Triple Sulfa	SSS	100	23	-	-	-	-
Ampicillin	Am	100	23	-	-	-	-
Kanamycin	K	100	23	-	-	-	-
Streptomycin	S	100	23	-	-	-	-
Penicillin G	P	100	23	-	-	-	-

Diarrheas caused by shigella vary from one country to another in accordance with culture, customs, and hygienic and social conditions. Studies so far conducted by WHO have shown that diarrheas caused by *Shigella sonnei* is less severe than that caused by *Shigella dysenteriae*. **it was also demonstrated that when** in a society hygienic and social conditions improve, diarrheas caused by *Shigella sonnei* becomes more prevailing than others. As reported by WHO, since

1920, infections caused by shigella, have been very rare in Europe, and North America. Until 1970, no major epidemic of this disease had been reported. Only 40 cases of this infection were reported in the US from 1970-1972. In 1976, an epidemic of *Shigella dysenteriae* was reported in Sri Lanka.³ The difference between salmonellosis and shigellosis is that in developed countries diarrheas caused by shigella is negligible in newborns below the age of 6 months and in

Table No 8- Study of Antibiotic Resistance Spectrum of *Salmonella typhimurium* Isolated from Diarrhea Cases of Children of 0-5 Years Age Group in Tehran, in Summer 1986.

Antibiotics Sulfonamides	Abbr.	Resistant		Intermediate Sensitivity		Sensitive	
		%	No	%	No	%	No
Furadahtin	Fd	-	-	-	-	100	17
Polymixin B	PB	-	-	-	-	100	17
Nalidixic Acid	NA	-	-	5.8	1	94.1	16
Cephalothin	Cf	11.8	2	17.6	3	70.5	12
Tetracycline	Te	70.5	12	-	-	29.4	5
Gentamycin	Gm	88.2	15	-	-	17.7	2
Neomycin	N	88.2	15	-	-	11.8	2
Chloramphenicol	C	88.2	15	-	-	11.8	2
Triple Sulfa	SSS	94.1	16	-	-	5.8	1
Ampicillin	Am	94.1	16	-	-	5.8	1
Kanamycin	K	94.1	16	-	-	5.8	1
Trimethoprim	SXT	94.1	16	-	-	5.8	1
Streptomycin	S	100	17	-	-	-	-
Penicillin	P	100	17	-	-	-	-

Table No 9 -Study of Antibiotic Resistance Spectrum of Shigella Isolated from Diarrhea Cases of Children of 0-5 Years Age Group in Tehran, in Summer 1986.

Antibiotics Sulfonamides	Abbr.	Resistant		Intermediate Sensitivity		Sensitive	
		%	No	%	No	%	No
Furadantin	Fd	-	-	-	-	100	5
Polymixin B	PB	-	-	-	-	100	5
Cephalothin	Cf	-	-	-	-	100	5
Neomycin	N	-	-	-	-	100	5
Nalidixic Acid	NA	-	-	-	-	100	5
Gentamicin		-	-	-	-	100	5
Kanamycin	K	-	-	20	1	80	4
Tetracycline	Te	20	1	-	-	80	4
Chloramphenicol	C	40	2	-	-	60	3
Ampicillin	Am	60	3	-	-	40	2
Triple Sulfa	SSS	80	4	-	-	20	1
Trimethoprim	SXT	100	5	-	-	-	-
Streptomycin	S	100	5	-	-	-	-
Penicillin	P	100	5	-	-	-	-

infants.¹³ Studies conducted in India has shown that shigella is the least common cause of diarrheas in children.¹⁴

Studies carried out in various cities of Iran, and the comparison of their results with the result of our study have shown that in Iran there has been a considerable decrease in the number of diarrheas caused by shigella, which has been replaced by diarrheas caused by salmonella. What is more important is that in Iran, *Shigella dysenteriae* is the microorganism most rarely

isolated in tests. For instance, in this very study, in 5 isolated cases of shigella, 3 cases were of *Shigella flexneri* type 6, and 2 of *Shigella sonnei* species.

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