ENTEROTOXIN PRODUCTION BY YERSINIA SPECIES AT 4 AND 25°C

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Abstract — Yersinia enterocolitica is new included in the classification of both the invasive bacteria (e.g. Shigella) and the toxicogenic bacteria (e.g. Escherichia coli). Three human strains and 43 environmental strains were used to produce Y. enteroclitica enterotoxin at 4°C , 25°C and 37°C using infant mouse assay. At 25°C enterotoxin was produced by 10 environmental strains and by all of the 3 human strains. Enterotoxin was produced by only 3 environmental strains (yersinia intermedia) at 4°C. At 37°C, none of the strains produced enterotoxin. This enterotoxin was stable for 2 months at 4°C and for 6 months at 60°C. At 100°C for 10 to 60 minutes and 121°C (autoclave) for 20 minutes this enterotoxin did not loss its activity. The results indicate that Y. intermedia may cause food intoxication after food storage at 4°C temperature.
Acta Medica Iranica 35 (3 & 4): 69-73; 1997

Key words: Yersinia; enterotoxin; diarrhoea

INTRODUCTION

In 1939 Schleifstein and Coleman introduced a gastrointestinal disease caused by Y. enterocolitica. A few years later, Mollaret found that Y. enterocolitica is among the main organisms capable of causing diarrhea in human (1-2).

In 1975 Carter (3) revealed a relationship between the induce infection of mice caused by Y. enterocolitica and the natural infection of humans.

At the time being, two pathogenic properties have been defined in Y. enterocolitica: (1) virulence (ie. the invasive function), which results in proliferation of the organisms in tissues, and (2) toxigenicity, marked by the ability of this organism to produce an enterotoxin similar to E. coli (4-6).

Production of enterotoxin after cultivation between 20 and 30°C in vitro is widespread among Yersinia spp. Enterotoxin production at the human body temperature has so far only been demonstrable for a group of non-sucrose fermenting strains (Y. kristnsenii) with uncertain clinical significance (7). Hence, the clinical importance of this enterotoxin is questionable.

In this study, our purpose was to determine the ability of Y. enterocolitica in producing an enterotoxin similar to that of E. coli in the laboratory conditions at various temperatures.

MATERIALS AND METHODS

Three Bacterial Strains

Two types strains were used in order to produce enterotoxin: (1) Human strains, which had been obtained from the pasteur's Institute in Paris from patients suffering from gastroenteritis. (2) Environmental strains which had been collected from water specimens (8), included 19 strains of Y. enterocolitica, 15 strains of Y. intermedia, 3 strains of Y. frederiksenii and 6 strains of Y. kristensenii. Their serological properties and source of isolation are shown in Table 1.

Culture Media

Okamoto proposed the CY media (9) which contains 2% casamino acid, 1% yeast extract, and 0.4% glucose and a PH=8.5 for culturing, and the production of enterotoxin.

Enterotoxin Production

The strains were initially cultured in 10 ml CY media and kept at 25°C for 24 hours. The culture was then added to a 500 flasks containing 100 ml CY media, and shaken at a rate of 200 rpm, for 48 hours, in a 25°C warm bath. After culturing, we centrifuged each culture at a rate of 8500 rpm for 30 minutes at 4°C. We filtered a part of the surface liquid with a 0.45 μm filter and the kept both the filtered liquid and the remainder of the surface liquid at-60°C. The same method of producing enterotoxin was used at 37°C for 48 hours, and at 4°C for 7 days (10).

Biologic Testing

For the suckling mouse assay, 0.1 ml of culture supernatant with 0.001% Evans blue dye as a marker was administered by gastric tube into the milk filled stomach of 3 to 5 day old suckling mouse. The suckling mouse were kept for 4 h at 25°C and then sacrified by inhalation of chloroform, and according to Deens' proposal (11). After which the entire intestines were removed, a ratio of intestine weight to remaining body weight (IW/BW) of > 0.083 was considered to be a positive response, and 1 unit was defined as the

minimum amount of protein required to give a positive response. The enterotoxin titer was expressed as the reciprocal of the highest dilution that gave one unit of the enterotoxin activity. Five mice were used for determination of the enterotoxin titer of each sample.

RESULTS

The presence of Yersinia spp. enterotoxin (ST) in culture filtrates was indicated for 10 (23.3%) of the 43 environmental strains of Y. enterocolitica and Y. intermedia and all of 3 human strains by infant mouse assay.

The macroscopic feature of the mouse gut is seen in figure 1. On the right side of this figure, the accumulation of water and electrolytes, and the inflammation of the gut is visible, which are due to the activation of guanylate cyclase focuses on enterotoxin production at various temperatures.

Table 1. At 25°C enterotoxin was produced by all the human and environmental strains. Enterotoxin was produced by only 3 environmental strains (Y. intermedia) at 4°C, and at 37°C, niether of the strains produced enterotoxin. The stability of enterotoxin in heat or cold is demonstrated in Table 2 and Table 3. The enterotoxin produced by human strains maintained their activity against mouse gut, although to a lower extent, after being kept at 4°C for 2 months and at -60°C for 6 months. This enterotoxin also maintained its activity against mouse gut after applying a 100°C heat for 60 minutes or a 121°C heat (autoclave) for 20 minutes to it.

Table 4 demonstrates the concentration of enterotoxin after being diluted. The maximum concentration of enterotoxin in environmental strains were obtained after being diluted 6 times and kept at 25°C for 48 hours. In human strains the same result was obtained after diluting the enterotoxin 8 times (at the same time period and the same temperature).

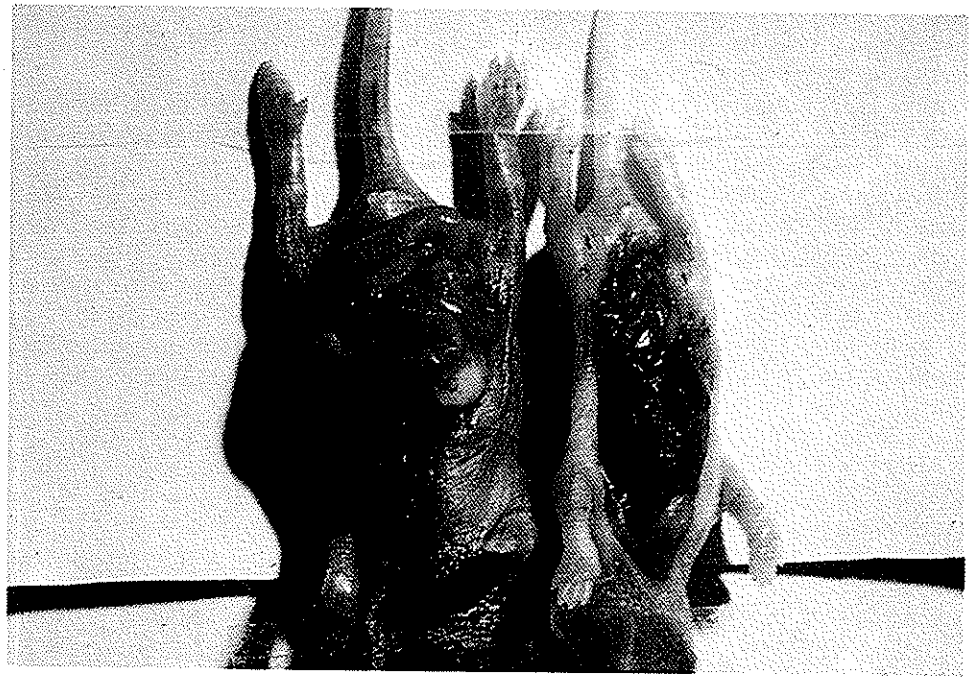

Fig. 1. The macroscopic appearance of a 4-5 day mouse
The right side: the Mouse gut, 4 hours after ingesting the surface liquid of the bacterial culture containing enterotoxin, which demonstrates the accumulation of clear fluid. The left side: the mouse gut, 4 hours after ingesting the surface liquid of the bacterial culture without enterotoxin. The dark color is due to the presence of bile in the intestine.

Table 1. Enterotoxin production by Y. enterocolitica and Y. intermedia indicated by the infant mouse assay

Strains	Source	Serogroup	Biotype	lysotype	Enterotoxin production at 4°C	25°C	37°C
human							
y.e	Gastroenteritis	3	4	VIII	-	+	-
"	"	"	"	"	-	+	-
"	"	"	"	"	-	+	-
Environmental							
a							
y.e	water	18	3	xo	-	+	-
y.e	"	AA	1	xz	-	+	-
y.e	"	AA	1	xz	-	+	-
y.e	"	AA	1	xz	-	+	-
b							
y.i	"	AA	1	xo	+	+	-
y.i	"	AA	4	xo	+	+	-
y.e	"	AA	1	xz	-	+	-
y.i	"	AA	1	xo	-	+	-
y.i	"	AA	4	xo	+	+	-
y.i	"	AA	4	xo	-	+	-

a: yersinia enterocolitica
b: yersinia intermedia

Table 2. Effect of various storage conditions on Y. enterocolitica crude toxin

human isolate	IW/BW ratio after storage: Fresh enterotoxin	(at 4°C) 2 week	(-60°C) 1 month	2 month	6 month
4052	0.095	0.092	0.087	0.083	0.085
6809	0.110	0.111	0.102	0.094	0.090
6810	0.106	0.105	0.098	0.087	0.088

a) IW/BW ratio: Mean of five determinations.
Ratios of 0.083 or greater are considered positive.

Table 3. Effect of heating on enterotoxigenic activity of Y. enterocolitica

Heat treatment	IW/BW ratio[a]
unheated	0.108 ± 0.007
100°C 10 min	0.108 ± 0.005
100°C 20 min	0.104 ± 0.005
100°C 40 min	0.099 ± 0.006
100°C 60 min	0.087 ± 0.004
121°C min (Autoclave)	0.085 ± 0.005

a) IW/BW ratio: Mean of five determinations ± standard error Ratios of 0.083 or greater are considered positive.

Table 4. Effect of growth temperature on enterotoxin production[a]

origin	Temp (oC)	Incubation period (hr)	b OD	Enterotoxin Titer
Environmental	4	48	0.788	d
"	4	7 day	5.52	3
"	25	24	7.34	4
"	25	48	8.57	6
"	37	24	1.20	-
"	37	48	2.25	-
human	4	48	0.574	-
"	4	7 day	3.90	-
"	25	24	6.55	5
"	25	48	9.34	8
"	27	24	5.54	4
"	27	48	7.70	6
"	30	24	4.83	-
"	30	48	5.12	4
"	33	24	2.12	-
"	33	48	2.30	-
"	37	24	1.34	-
"	37	48	1.52	-

a) Enterotoxin activing was assayed in infant mice.
b) Optical density (OD) of cultures as measured in a spectrophotometer at 600 nm.
c) Reciprocal of the highest dilution that gave a positive result in infant mice assay.
d) No enterotoxin activity in undiluted culture filtrates.

DISCUSSION

In various studies, it has been shown the Y. entercolitica strains are capable of producing an enterotoxin similar to that of E. coli (4-6).

In our studies, we examined 3 human strains derived from children suffering diarrhea, and 43 environmental strains which were collected before (8), in order to produce enterotoxin at different temperatures.

At low temperatures (4°C) 3 environmental strains of Y. intermedia produced enterotoxin after 7 days. Kapperud (12) demonstrated that serotypes 0:11 and 0:28 of environmental strains of Y. Kristensenii could produce enterotoxin at low temperatures; but neither of the human strains produced enterotoxin at 4°C.

At 25°C, 5 strains of Y. enterocolitica and 5 strains of Y. intermedia, all of which were obtained from the environment, produced enterotoxin. All the 3 human strains had the ability of producing enterotoxin at this temperature.

At 37°C, Neither of the strains produced enterotoxin. Nevertheless, kapperud (10) indicated that serotypes 0:11 and 0:28 of environmental strains of Y. Kristensenii produced enterotoxin at 37°C. Neither of the strains of Y. kristensenii in our study could produce enterotoxin in various temperatures. Studies performed on a temperature range of 25-37°C show that enterotoxin can only be produced at temperatures lower than 30°C (12-13).

The optimum condition for producing enterotoxin is incubation for 48 hours at 25°C where the maximum optic density (OD) and the maximum concentration of enterotoxin is observed. Enterotoxin production by Y. enterocolitica was demonstrable only when the organisms were grown at 30°C or below. The comparison of cell concentrations and the amount of toxin produced in cultures incubated at various temperatures suggests that the growth of these organisms was favored at the lower temperatures resulting in the accumulation of enterotoxins in culture supernatant fluids to a level detectable by the available assay techniques.

After storage of culture supernatants at 4°C for 2 months and 60°C for 6 months did not inactivate the crude Toxin. Similar results were published by several authors (9,14,15).

Most of the pathogenic bacteria (in humans) are mesophilic. Yersinia genus and especially Y. enterocolitica, have the ability to grow at the low temperature of 4°C, which is responsible for the increased cases of yersiniosis due to the preservation of food in cool places after the 1960.

The variety of the pathogenicity of Y. enterocolitica in relation to the preservation temperature has been shown by Nilehn (16). If Y. enterocolitica, previously cultured at 37°C, is injected into the mouse peritoneum, it will be excreted rapidly, but if it was previously cultured at 25°C, it is able to grow in the peritoneum.

The stability of this toxin at 100°C for 60 minutes and at 121°C autoclave for 20 minutes (17), reflects the importance of prevention and controlling contamination of food by this bacteria, both in house holds and in food industries. The production of enterotoxin has been seen only in 24 and 48 hours cultures accompanied with modest shaking. This shaking provides optimal aeration for bacterial growth and enterotoxin production in the culture media. The results obtained by Velin (18) about enterotoxin production in meat and sausage confirms our opinion concerning aeration in enterotoxin production.

Some reports (19) indicate the inhibitory effect of glucose in the production of enterotoxin by E. coli. However, in our study, the addition of 0.4% glucose to culture media, not only didn't the production of heat-stable enterotoxin by Y. enterocolitica, but also increased it (data not shown).

It has been shown in various investigations that enterotoxigenic strains of E. coli, have the ability to produce heat - stable (ST) enterotoxin in vivo and in vitro in animals and humans (17,20) where as the inability to produce enterotoxin in vitro at 37°C in our and other (13,21) studies, and the lack of relationship between Yersinia induced diarrhea and enterotoxin production in animals in the study performed by Pai (22,23), makes the role of heat - stable Yersinia enterotoxin in causing diarrhea uncertain.

At present, it would be better to classify Y. enterocolitica as the intestinal pathogens that cause colitis and acute enterocolitis due to invasion.

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