Volume 6 Número 4 Out.-Dez. 1975

Revista de Microbiologia

Órgão Oficial da Sociedade Brasileira de Microbiologia
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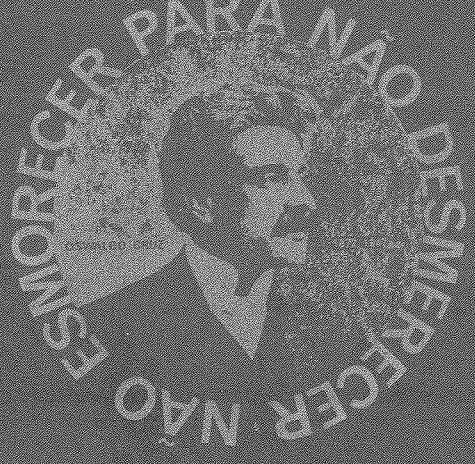

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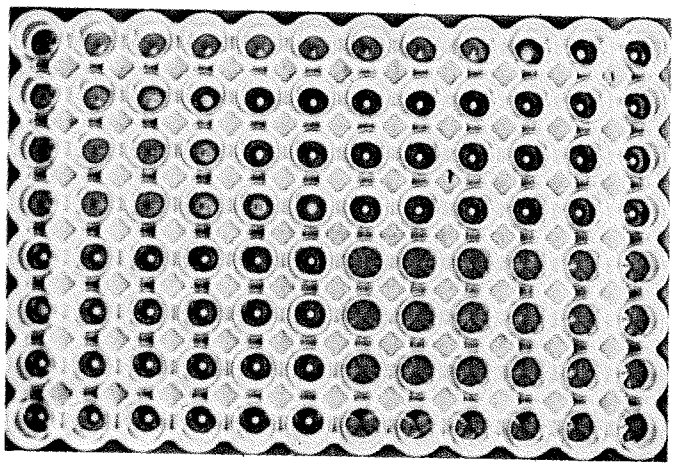

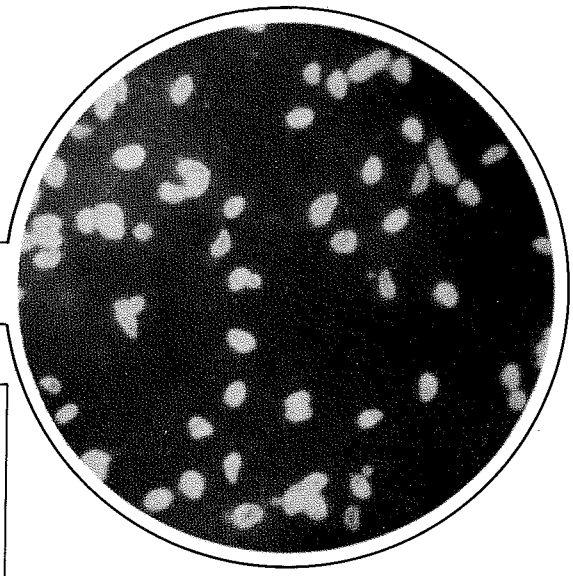

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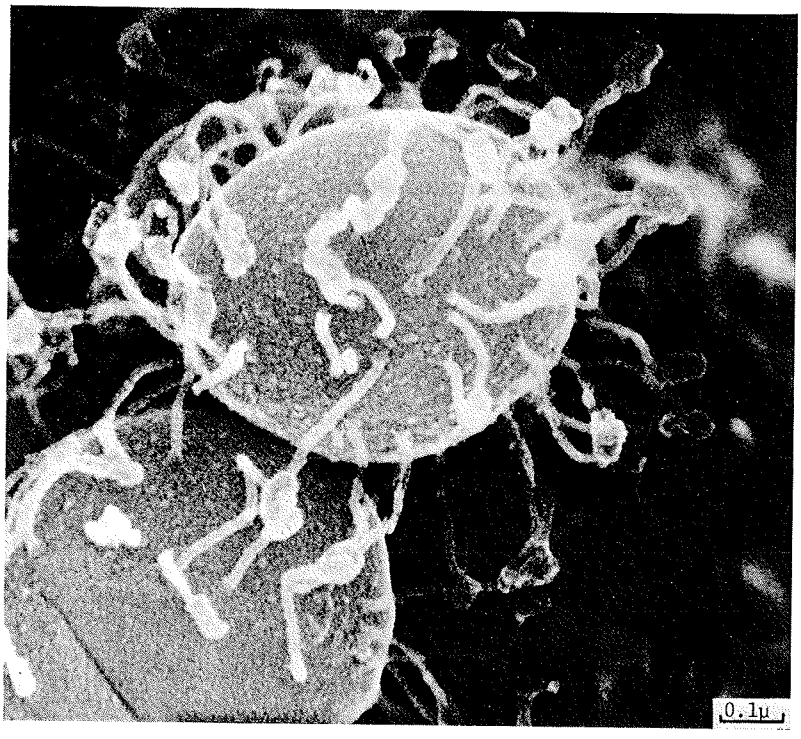

Imagem eletrônica secundária de fago PØ55 adsorvido sobre *Staphylococcus*, obtida com JEM-100C, equipado com SEG e ASID.

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Studies on carbon and nitrogen sources for commercial production of bacterial alpha amylase*

S.A.Z. Mahmoud**, S.M. Taha, R.M. Attia & S.H. El-Gammal

Summary

Alpha amylase is extensively used in testile manufacture which is one of the main industries in the A.R.E. It was found of interest to study the economical carbon and nitrogen sources that secure the maximum yield of this enzyme industrially. Several carbon and nitrogen sources viz, rice bran, enzymatically hydrolysed corn starch, commercial dextrin for carbon sources and brewery yeast, baker's yeast, malt sprout, imported and local corn steep liquor and gluten were investigated. Results showed that rice bran (10%) is superior than any carbon source used. In view of the high amounts of impurities and troubles implicated in other processes of filtration and extraction it was found that 3% corn starch supplemented with 2% gluten as nitrogen source secured the best economical and industrial yield of alpha amylase.

Resumo

Estudos sobre fontes de carbono e nitrogênio para a produção comercial de alfa-amilase bacteriana

Definição de um meio de cultura adequado, do ponto de vista econômico e industrial, para a obtenção de alfa-amilase por processo fermentativo. Foram verificadas fontes de carbono como farelo de arroz, hidrolisado enzimático de amido de milho e dextrina comercial, e fontes de nitrogênio, como levedura de cerveja, do pão, germe do malte, água de milho e glúten. Os resultados mostraram que o farelo de arroz (10%) é superior a qualquer outra das fontes usadas. Porém, esse substrato revelou-se muito susceptível a contaminações, além de apresentar grande quantidade de impurezas. O meio contendo 3% de amido de milho e 2% de glúten é proposto como sendo o melhor em rendimento enzimático.

Introduction

The economics of enzyme fermentation limit the carbohydrate and nitrogen sources to cheap industrial and agricultural waste materials. Purified ingredients, although readily fermentable, are too costly for the production with economical yield.

Several investigators pointed out the importance of carbon and nitrogen sources in the production of amylases[2, 3, 5, 7, 8]. The current study presents the utilization of local industrial and agricultural wastes as carbon and nitrogen sources on amylase production by *B. subtilis* S-3217[6].

Material and Methods

The medium of Cadmus & col.[1] modified by Mahmoud, Attia & El-Gammal (unpublished date in El Nasr Pharm. Chem. Co) was used for commercial amylase production. It consists of 10% rice bran, 1.5% corn steep liquor, 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$ and 0.001% each of $MnSO_4$, $Fe_2(SO_4)_3$ and NaCl at pH 6.5.

The amylase activity was determined according to the method of Standete, Kneen & Blish modified[4].

Fermentation was carried out in $10M^3$ stainless steel fermentors (jacketted with turbine agitator variable speed drive, bubbler pipe for blowing over and electric motor), using $6M^3$ of the medium. The fermentor was supplied with stirrer at 180 r.p.m.. Sterile air was injected at the rate of 0.5/ℓ. The fermentation was carried out at 30°C for 72 hours.

Results

Effect of carbon sources — When using rice bran as carbon source in the scale production unit, considerable difficulties were observed in obtaining non

* Presented during GIAM IV — Fourth International Conference on the Global Impacts of Applied Microbiology, São Paulo 23–28 July 1973.

** Faculty of Agriculture, Ain Shams University, Cairo, Egypt, A.R.E.

contaminated substrate. Special treatments of cooking and sterilizing the mash should be undertaken. Piping off the fermented liquor and other processes constituted difficult problems. In addition, during the cooking of rice bran the fine particles settled on the walls above the water line were cooked into hard scales, providing a probable source of contamination; besides dust and soil particles contained in this ingredient which harbour high densities of microorganisms. To overcome these difficulties, it was necessary to use other ingredients with less troubles.

Enzymatically hydrolysed corn starch (EHCS) was investigated. To liquefy the thick mash of corn starch, ground malt was added. Initially the malt was added to the slurry into the cooker (0.5%) and the temperature maintained at 60°C for 20 minutes.

Results in Table 1 show that rice bran (RB) used as control was in general superior to EHCS. When using 2 and 4% EHCS nearly similar activities during the fermentation period was observed. Increasing the concentration of EHCS gave significantly lower activities. This may be due to the lower nitrogen content in the medium, which can balance such a high increase in carbon.

Table 1

Effect of substituting rice bran (RB) by enzymatically hydrolysed corn starch (EHCS)

EHCS %	Amylase activity (NU/ml) Fermentation period (day)		
	1	2	3
Control (RB)	440	6100	8200
2	2800	3300	3800
4	2400	3500	4000
6	2400	2000	3000

Previous results showed that corn starch hydrolysed by malt diastase was inferior to rice bran as carbon source. This may be due to the action of malt diastase which split starch granules rapidly giving high amount of maltose and lower amount of dextrin. Such end products might influence the alpha amylase production. This is besides the comparatively low nitrogen content of the medium.

When dextrin was used as carbon source (Table 2), the best concentration of dextrin giving the highest (optimal) activity was 3.5%. Higher concentrations, however, showed no significant differences in activity. This may be attributed to the high carbon content which disturbe the C/N ratio of the medium.

Results also show that the control (RB) gave higher activity than 3.5% dextrin treatment. This was attributed to the higher percentage of impurities in the liquor of rice bran than in dextrin medium. In fact, high amount of impurities was obtained after the filtration of the rice bran liquor that left exceedingly lower volume of fermented liquor than in the case of dextrin. This can be determined by calculating the units of the enzyme/volume before and after filtration of rice bran and dextrin treatments.

When substituting rice bran by different carbon sources, it was necessary to balance the C/N ratio of the medium by increasing the nitrogen content since rice bran, although used as carbon source, yet contained a high nitrogen level. To overcome the high C/N ratio given by the corn starch, the medium was enriched with the addition of gluten to maintain a narrower C/N ratio.

Results in Table 3 show that the optimum concentration of corn starch, which gave the highest yield of enzyme, was 3% supplemented with 1% gluten as additional nitrogen source. The lowest corn starch treatment, i.e. 2%, gave nearly the same activity as in the control.

Table 2

Effect of substituting rice bran (RB) by commercial dextrin

Dextrin %	Amylase activity (NU/ml) Fermentation period (day)		
	1	2	3
Control (RB)	5000	7200	7500
3.0	4000	6000	6000
3.5	3400	6300	6500
4.0	2600	5400	5400
5.0	300	6000	6000

Table 3

Effect os substituting rice bran by corn starch and gluten

Treatment	Ingridient %				Amylase activity (NU/ml) Fermentation period (hr)		
	RB	Corn starch	Gluten	CSL	24	48	72
Control	10	0	0	1.5	5300	7500	9000
1	0	2	1	1.5	3500	6500	8500
2	0	3	1	1.5	4500	11000	11000
3	0	4	1	1.5	2800	5500	7500

Effect of nitrogen sources

Nitrogen sources should be less expensive, locally produced and at the same time adequate to satisfy the nutritional requirements of the organism tested. Several nitrogen sources were tried viz: brewery yeast, baker's yeast, malt sprout, corn steep liquor and fermentol.

The optimum concentration of brewery yeast after several laboratory experiments was 7%. The activities in the five replicates (Table 4) ranged from 5500-7150 NU/ml after 72 hour. This variation in activity can be attributed to the concentration of the yeast in the brewery liquor. This was not constant, since the concentration of yeast cells varied from one batch to another. The fact that brewery yeast does not contain standard amount of yeast cells, transportation of the liquor is expensive and, finally, its preservation is difficult wich does not favour its use in an industrial scale.

Table 4

Effect of 7% brewery yeast

Replicate Nº	Amylase activity (NU/ml) Fermentation period (hr)		
	24	48	72
1	400	4500	5500
2	400	5300	5500
3	800	5500	5500
4	1100	4700	7150
5	1000	4400	6100
Average	540	4880	5950

Baker's yeast was used to replace brewery yeast because of its relative cheapness, availability, facility in transportation and preservation. All this favoured its use as a nitrogen source. Results in Table 5 show that 4% baker's yeast can be used as a good cheap nitrogen source. The percentage (4%) had been previously found to be the most economical concentration after several laboratory trials. Activities were generally consistant and higher than that observed when using brewery yeast.

When malt sprout was used as a nitrogen source, it was found that baker's yeast treatment was more superior in this respect. Higher rate of amylase activity was achieved when using 4% baker's yeast as nitrogen source than all concentrations of malt sprout. Comparing to malt, it was found that 6 and 8% gave nearly the highest activities recorded during the experimental period. In fact there were no differences in amylase activities between 6 and 8% malt sprout treatments. The only difference in activities was recorded after 48 hours (Table 6).

Corn steep liquor (CSL) is another cheap raw material, used frequently as nitrogen source in many fermentation industries. Results recorded in Table 7 show that 1.5% was the most suitable and economical concentration which gave the highest yield.

Table 5

Effect of 4% baker's yeast on the production of bacterial alpha amylase

Replicate Nº	Amylase activity (NU/ml) Fermentation period (hr)		
	24	48	72
1	1000	2200	7000
2	400	1750	7000
3	1000	2000	6500
4	600	4500	9500
5	250	2400	7500
Average	650	2570	7500

Table 6

Effect of substitution of baker's yeast with malt sprout, on the production of alpha amylase

Source %	Amylase activity (NU/ml) Fermentation period (hr)		
	24	48	72
Malt sprout			
2	400	1000	1000
4	500	2300	3500
6	600	2600	4400
8	700	2400	4400
Baker's yeast			
4	1100	3500	7400

Table 7

Effect of substituting baker's yeast with CSL

Source %	Amylase activity (NU/ml) Fermentation period (hr)		
	24	48	72
CSL			
1	500	1800	2000
1.5	1150	5200	9500
2	1200	4500	9000
2.5	1000	5000	8000
3	1100	5000	8500
Baker's yeast			
4	800	2000	6500

It was conclued' from the results that CSL is superior to brewery yeast, baker's yeast and malt sprout in the production of bacterial alpha amylase.

Fermentol, the trade name of CSL precipitate, produced locally in A.R.E. was investigated as a nitrogen source. Results in Table 8 show that the best concentration was 1.0%. It gave 7500 unit after 72 hours, while the CSL treatment gave 7000 unit.

Table 8

Effect of substituting CSL by fermentol

Source %	Amylase activity (NU/ml)		
	Fermentation period (hr)		
	24	48	72
Fermentol			
1	1800	6000	7500
1.5	2100	6260	7500
2	1750	5600	7400
CSL			
1.5	2000	5200	7000

This means that the local precipitate is superior to corn steep liquor.

Gluten, another cheap raw material produced locally was used in this investigation. Results in Table 9 show that 2% gluten is superior in the production of amylase. It can replace the CSL in the composition of the media.

From the afore-mentioned results, it could be concluded that using 3% corn starch as carbon source and 2% gluten as nitrogen source gave the best economical yield of bacterial alpha amylase on the commercial scale.

Table 9

Effect of substituting CSL with gluten

Gluten %	Amylase activity (NU/ml)		
	Fermentation period (hr)		
	24	48	72
2	500	8000	12500
4	400	5900	11250
5	300	4000	10000
6	300	3700	7500

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Effect of some carbon and nitrogen sources on the production of 2,3-butanediol*

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Summary

Bacillus subtilis (S-58), *Bacillus polymyxa* (S-69) and *Aerobacter aerogenes* (F-343), local strains, were studied with regard to carbon and nitrogen influencing the production of 2,3-butanediol. 1 — Proteosepeptone was the best nitrogen source for glycol production by the three test organisms. 2 — The best nitrogen concentration for the glycol production in the fermentation media proved to be 0.07% for the three test organisms. 3 — Carbon sources giving the best growth and highest glycol yield were sucrose for *B. subtilis*, galactose for *B. polymyxa* and glucose for *A. aerogenes*. 4 — Carbon concentrations that gave the highest glycol yields were 7, 3 and 10% for *B. subtilis, B. polymyxa* and *A. aerogenes* respectively. Economically 5% carbon for *B. subtilis* and *A. aerogenes* and 3% carbon for *B. polymyxa* were the best carbon concentrations.

Resumo

Efeito de algumas fontes de carbono e nitrogênio na produção de 2,3-butanediol

Estudo de variações na produção fermentativa de 2,3 butanediol por *Bacillus subtilis* (S-58), *B. polymyxa* (S-69) e *Aerobacter aerogenes* (F-343), frente a variações nas fontes de carbono e nitrogênio. Verificou-se que o melhor rendimento em 2,3 butanediol era alcançado, usando-se, como fonte de nitrogênio, a proteosepeptona (0,07%) para os três microrganismos testados. Em relação à fonte de carbono, as melhores foram sacarose (7%), para *B. subtilis*, galactose (3%), para *B. polymyxa*, e glicose (10%), para *A. aerogenes*. Em termos de rendimento econômico, as melhores concentrações em carbono foram de 5%, para *B. subtilis* e *A. aerogenes*, e de 3%, para *B. polymyxa*.

Introduction

It is difficult to make a general statement regarding the optimum concentration of carbon and nitrogen for the butanediol fermentation due to the variety of raw materials employed. In most studies, however, it seems that the usual concentrations employed were in the range of 5-10%[1,2,13,15]. Several investigators pointed out the importance of nitrogen sources in the production of glycol[1,4,5,6,13,14,18]. They also studied the effect of a number of carbon sources on the production of glycol and found that sucrose, beet molasses, black strap molasses and citrus molasses were satisfactory.

The current investigation presents the effect of different carbon sources on glycol production by *B. subtilis, B. polymyxa* and *A. aerogenes*, locally isolated by the authors from soil[17].

Materials and Methods

B. subtilis (S-58), *B. polymyxa* (S-69) and *A. aerogenes* (F-343) which were found from previous investigation[17] to give maximum yield of glycol were used as test organisms.

The glucose-yeast extract broth[9,10] was used for testing the production of glycol by *B. subtilis*. It consists of 3% glucose, 1% yeast extract and 1% $CaCO_3$. The starch yeastextract broth[5] was used for *B. polymyxa*. It consists of 2% soluble starch 0.5% yeast extract and 1% $CaCO_3$. The glucose urea broth (20) was used for *A. aerogenes*. It consists of 5% glucose, 0.06% KH_2PO_4, 0.025% $MgSO_4 . 7H_2O$, 0.5% $CaCO_3$ and 1% urea (20%).

The method of Morris[8] was used to determine quantitatively the residual carbohydrates as glucose. The method of Nelson[11] modified by Somogyi[16] was

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used to determine the reducing sugars as glucose. The microdiffusion method of Winnick[21] was used for glycol determination.

Results and Discussion

The nutrition of all cells supports two fundamental functions: the generation of energy and the synthesis of protoplasm. The environment in which the organism grows must supply the elements needed in a form available to the cell[12]. These two functions depend on a complex network of chemical reactions brought about by specific enzymes. The synthesis of enzymes is a continuous process in every living organism. An increase in the number of molecules of each essential enzyme must clearly take place whenever the quantity of living matter increases during growth. But even if growth is not taking a continuous, synthesis is needed to replace the enzyme molecules which have undergone destruction. The formation of enzymes usually takes place within living cells and is influenced by the environment or composition of the medium surrounding the cells. The two main nutritional sources, i.e. nitrogen and carbon providing these requirements were studied. Different nitrogen sources were added to the basal media in amounts calculated to give final nitrogen concentration of 0.12%, 0.06% and 0.09% (as those present in the basal media), as determined by micro-Kjeldahl for *B. subtilis, B. polymyxa* and *A. aerogenes* respectively.

Table 1

Effect of nitrogen source on the production of glycol by the three test organisms

Nitrogen source	Diol yield (g/100ml culture)		
	B. subtilis	*B. polymyxa*	*A. aerogenes*
Urea	0.41	0.20	1.51
Proteosepeptone	1.00	0.53	1.61
Peptone	0.94	0.42	1.23
Casein	0.42	0.30	0.42
Yeast extract	0.89	0.42	1.46
Gelatin	0.30	0.27	0.40

Results in Table 1 shows that the source of nitrogen greatly affects the butanediol production. In general proteosepeptone gave the highest diol yield for the three test organisms. This was followed in decreasing order by peptone, yeast extract, casein, urea and gelatin for *B. subtilis*; peptone, yeast extract, casein, gelatin and urea for *B. polymyxa;* and urea, yeast extract, peptone, casein and gelatin for *A. aerogenes*.

It can generally be concluded from these results that proteose peptone was the best nitrogen source for the production of diol. The reason for such a result could be due to the chemical constitution of the nitrogen source and the ratio of amino acids present, which may affect the formation of enzyme responsible for the diol fermentation.

It is shown in Table 2 that 0.07% nitrogen was the optimum concentration used for the diol production by the three test organisms, giving 0.97, 0.57 and 1.51% of diol for *B. subtilis, B. polymyxa* and *A. aerogenes* respectively.

Table 2

Effect of nitrogen concentration on the production of diol by the three organisms

Nitrogen Conc.* (%)	Diol yield (g/100ml culture)		
	B. subtilis	*B. polymyxa*	*A. aerogenes*
0.035	0.92	0.25	1.24
0.070	0.97	0.57	1.51
0.140	0.97	0.57	1.51
0.210	0.97	0.57	1.51
0.280	0.97	0.57	1.51

* Proteosepeptone

Different carbon sources[10] were tried. The amounts of carbon compounds added to the media were calculated to give a final concentration of 1.2%, 0.7% and 2.0% for *B. subtilis, B. polymyxa* and *A. aerogenes* respectively. Thus the amount of carbon was fixed for each test organism regardless of the source to eliminate errors which might occur as a result of difference in carbon concentration in each source.

Results in Tables 3, 4 and 5 indicate that sucrose gave the best growth and maximum yield of diol when *B. subtilis* was used. Concerning the conversion

Table 3

Efect of carbon source on the production of diol by *B. subtilis*

Carbon source	Sugar consumed (%)	Diol yield		
		g/100ml culture	% Conversion value on basis of	
			Sugar	Carbon
Glucose	92.0	1.02	36.9	48.9
Galactose	90.0	0.88	31.8	42.2
Fructose	95.0	0.87	30.7	40.6
Sucrose	96.0	1.06	36.9	48.9
Lactose	2.7	0.08	10.0	13.4
Maltose	5.3	0.33	20.6	27.2
Starch	5.0	0.10	6.3	8.4
Dextrin	4.7	0.10	6.8	8.9
Mannitol	–	0.61	–	–
Glycerol	–	0.19	–	–

value on sugar and carbon basis, sucrose and glucose gave the highest yields. Other carbohydrates gave lower yields.

It can be concluded that sucrose and glucose greatly influenced the production of glycol by *B. subtilis*. These findings are in agreement with those obtained by several investigators[1, 13] who used glucose as a sole source of carbon in the production of glycol by *B. subtilis* (Ford's strain).

Starch gave the best growth for *B. polymyxa* as can be shown from the sugar consumption (Table 4). Concerning the conversion value on sugar base, sucrose showed maximum yield of glycol (53.6%).

Table 4

Effect of carbon source on the production of diol by *B. polymyxa*

Carbon source	Sugar consumed (%)	Diol yield		
		g/100ml culture	% Conversion value on basis of	
			Sugar	Carbon
Glucose	87.0	0.53	30.6	40.5
Galactose	91.0	0.68	37.2	49.2
Fructose	32.0	0.29	45.8	63.1
Sucrose	53.0	0.57	53.6	71.0
Lactose	25.0	0.08	16.0	21.5
Maltose	81.5	0.54	32.8	43.6
Starch	93.0	0.62	33.4	44.3
Dextrin	75.0	0.55	36.3	48.1
Mannitol	–	–	–	–
Glycerol	–	–	–	–

Lactose gave the best growth for *A. aerogenes* as can be seen from sugar consumption (Table 5). It was followed by glucose, maltose, galactose, fructose, dextrin, sucrose and starch.

Table 5

Effect of carbon source on the diol production by *A. aerogenes*

Carbon source	Sugar consumed (%)	Diol yield		
		g/100ml culture	% Conversion value on basis of	
			Sugar	Carbon
Glucose	85.0	1.38	32.0	42.9
Galactose	84.5	1.25	29.5	39.9
Fructose	66.2	1.03	31.0	41.1
Sucrose	46.0	1.16	50.6	66.9
Lactose	90.0	0.09	1.9	2.5
Maltose	85.0	0.65	15.4	20.4
Starch	46.0	0.00	00.0	0.0
Dextrin	50.0	0.17	6.8	9.1
Mannitol	–	0.41	–	–
Glycerol	–	0.28	–	–

The glycol yield was high when glucose was used as a sole source of carbon, followed in descending order by galactose, sucrose, fructose, maltose, mannitol, glycerol and lactose. Starch gave no yield of glycol when used as a sole source of carbon.

Concerning the conversion value on sugar basis, sucrose was the best carbon source for glycol production. The same trend was obtained when calculated on carbon base. These findings are in agreement with several investigators[1, 3, 7, 13, 14] who used glucose and sucrose for glycol production by *B. polymyxa* and *A. aerogenes*.

It has been reported that increasing the initial sugar concentration increases the yield of 2,3-glycol in bacterial fermentation[1, 3, 13]. Since it was found in the previous experiments that sucrose, galactose and glucose gave the maximum yields of glycol when used as sole carbon sources for *B. subtilis, B. polymyxa* and *A. aerogenes* respectively, an experiment was designed to study the effect of different concentrations of these sugars on the production of butanediol.

Results in Tables 6, 7 and 8 show that sugar concentration of 7% gave the highest glycol yield in case of *B. subtilis*. On the other hand 3% sugar concentration gave the best growth as can be seen from sugar consumption which reached 96.1%. Concerning the conversion values on sugar and carbon basis, 5% sugar concentration gave the highest economical yield (Table 6). In the case of *B. polymyxa* (Table 7), sugar concentration of 3% gave the highest glycol yield. Concerning the conversion value on sugar and carbon basis, 3% sugar concentration gave the highest economical yield. In the case of *A. aerogenes* (Table 8) the highest glycol yield was achieved when 10% sugar concentration was used. On the other hand 3% sugar concentration gave the best growth as can be seen from sugar comumption which reached 97.7%. Concerning the conversion values on sugar and carbon base, 5% sugar concentration gave the highest economical yield.

Table 6

Effect of different concentration of sucrose on the production of butanediol by *B. subtilis*

Sugar conc. (%)	Sugar consumed (%)	Diol yield		
		g/100ml culture	% Conversion value on basis of	
			Sugar	Carbon
1	93.3	0.23	20.9	27.6
2	94.6	0.58	25.2	33.6
3	96.1	0.97	27.9	37.0
5	93.7	2.03	36.2	47.9
7	88.1	2.44	33.0	43.7
10	75.0	1.83	20.4	27.0

Table 7

Effect of different concentration of galactose on the production of butanediol by *B. polymyxa*

Sugar conc. (%)	Sugar consumed (%)	Diol yield		
		g/100ml culture	% Conversion value on basis of	
			Sugar	Carbon
0.5	85.7	0.05	16.7	21.6
1.0	92.9	0.19	29.2	38.9
2.0	75.7	0.44	41.4	54.9
3.0	48.7	0.84	57.7	76.5
5.0	42.9	0.84	46.2	74.5
7.0	33.9	0.84	50.8	67.2

Table 8

Effect of different concentration of glucose on the production of diol by *A. aerogenes*

Sugar conc. (%)	Sugar consumed (%)	Diol yield		
		g/100ml culture	% Conversion value on basis of	
			Sugar	Carbon
1	97.0	0.26	26.4	34.9
3	97.7	0.90	31.0	40.7
5	79.6	1.30	32.8	43.3
7	62.4	1.31	30.1	39.8
10	50.0	1.39	27.8	36.8

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Multirresistencia en *Shigella* mediada por factores "R" que incluyen el determinante de resistencia para ampicilina-carbenicilina*

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Resumen

En Venezuela el 84% de las shigelas muestran resistencia múltiple. En 1972 la resistencia a ampicilina hace su aparición. Para Junio 1973 en 344 cepas aisladas, 11 (3%) muestran dicha resistencia cuyas características son estudiadas. Las cepas que han desarrollado resistencia a ampicilina, pertencen a los grupos B (serotipos B_1, B_2, B_4) y D. La resistencia a ampicilina, al igual que el resto de la resistencia observada en estas cepas, es transferible por conjugación a una *E. coli*. En dos de ellas constituye un factor "R" cuyo único determinante de resistencia es el de ampicilina. En las restantes, este determinante crea un estado hetero-R al conservar su individualidad episomal o se incorpora a los determinantes de resistencia que integran factores "R" más complejos que median multirresistencia. En dos cepas se originan así un patrón de resistencia múltiple sulfa-streptomicina-tetraciclina-cloramfenicol-kanamicina--neomicina-ampicilina-carbenicilina, de tal magnitud en su complejidad que solo ha sido descrito previamente en dos cepas de *Shigella*. No se evidencia segregación de genes de resistencia para ampicilina-carbenicilina. Los niveles de resistencia mediados por estes factores "R" son clinicamente significantes. En un paciente la resistencia a ampicilina se adquiere probablemente, en el intestino por conjugación, ante la presión selectiva impuesta por su uso, desde miembros de la flora residente que portaban factores "R" con determinantes de resistencia para ampicilina. En Venezuela a medida que el uso de ampicilina se incremente la shigelosis por cepas ampicilina resistentes aumentará en frecuencia e importância.

Summary

Multiresistance in Shigella mediated by R factors including that determinating resistance to ampicillin--carbenicillin

In Venezuela, 84% of *Shigella* strains show multiple resistance. In 1972 the *Shigella* strains developed resistance to ampicillin. By June 1973 in a study of 344 strains, 11 (3%) show such resistance which is characterized in this paper. The strains that have developed resistance to ampicillin belong to group B (serotypes B_1, B_2, B_4) and D. The resistance to ampicillin and the rest of the resistance observed in these strains are transferable by conjugation. In two strains a R factor is originated whose only determinant of resistance is ampicillin. In the remaining their determinants either create a Hetero-R state, if they maintain their episomal individuality, or join other determinants of resistance that are building up complex R factors that mediate multiresistance. Two strains originate a pattern of multiple resistance, sulfanilamides streptomycin-tetracycline-chloramphenicol-kanamycin-neomycin-ampicillin-carbenicillin, which has only been described before in two *Shigella* strains. The genes that mediate resistance to ampicillin are apparently the same that mediate carbenicillin resistance, because of the lack of genes segregation for these antimicrobial agents. The levels of the resistance mediated by these R factors are clinically significant. Resistance to ampicillin is acquired by one of the patients, due to the selective pressure imposed by its use, probably in the intestinal tract by conjugation where some members of the residential flora were bearers of R factors with determinants of resistance to ampicillin. It is expected that in the proportion in wich the use of ampicillin is increased in this country, the shigellosis by strains with resistance to ampicillin will also increase in frequency and importance.

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Introducción

Shigelas con patrones de multirresistencia fueron reportadas por primera vez desde Japón en 1955, poco tiempo después de la introducción de la streptomicina, tetraciclina y cloramfenicol en el tratamiento de procesos infecciosos. Desde entonces la frecuencia de aislamiento de cepas de *Shigella* con tal resistencia ha ido en aumento.

Este tipo de resistencia resultó estar mediada por elementos genéticos extracromosómicos de naturaleza episomal hoy conocidos como factores "R"[1, 2, 9, 10, 15, 16, 30, 35, 43, 44, 48, 55, 56].

La multirresistencia determinada por factores "R" en *Shigella* ha sido estudiada exhaustivamente en Japón. También ha sido reportada desde otros paises como: África, Rumania, Inglaterra, Francia, U.S.A., México, Guatemala, Brasil y Venezuela. De estos estudios se deduce que en las diferentes áreas geográficas las shigelas adquieren con frecuencia patrones de resistencia múltiple y además que existen variaciones en relación al desarrollo de resistencia para un determinado agente antimicrobiano[5, 12, 13, 17, 22, 24, 27—29, 31, 34, 36—40, 45—47, 50—52, 57—58].

Para la ampicilina (α-amino bencil penicilina) una penicilina semi sintética de adquisición relativamente reciente y que en la actualidad es considerada el antibiótico de elección para el tratamiento de la shigelosis[18, 23, 33], el desarrollo de resistencia ha sido reportado con frecuencia en shigelas aisladas en el continente europeo e infrecuentemente en aquellas aisladas en el Japón[13, 27, 36, 39, 50, 52].

En U.S.A. en diferentes estudios sobre *Shigella* se encuentran porcentajes de resistencia para este agente, los cuales oscilan entre 3% y 12%[18, 19, 22, 28]. Para el año 1970, en el resto de la América aún no se reportan cepas resistentes a ampicilina, a excepción de una aislada en Perú en el transcurso del citado año y estudiada por Carpenter en el Dysentery Reference Laboratory, London en Inglaterra[24, 34, 38].

En Venezuela, Prieto en el año 1969 no reporto resistencia para ampicilina en estudios realizados con 58 cepas de shigelas aisladas durante los años 1968—1969, ello a pesar de que en el 98% de las cepas se pudo evidenciar la existencia de resistencia múltiple y los porcentajes de resistencia para otros antimicrobianos fueron elevados: sulfa y streptomicina 95%, tetraciclina 79%, cloramfenicol 76% y kanamicina 24%[40].

La resistencia a la ampicilina hace su aparición en cepas de *Shigella* en el transcurso del año 1972.

Entre los años 1968 al 1973 en 344 cepas estudiadas, 11 de ellas (3%) muestran dicha resistencia.

En el presente trabajo se describen las características que asume la resistencia recientemente adquirida por las shigelas para este agente antimicrobiano.

Material y Metodos

Se estudian las características de la resistencia a los agentes antimicrobianos incluyendo ampicilina, en once cepas de shigelas aisladas en la localidad de niños con clínica de disentería bacilar en el curso de los años 1972—1973.

La metodología utilizada para su aislamiento del coprocultivo e identificación, bioquímica y serología es la descrita por Edwards & Ewing[14].

El método del disco de alta potencia según los criterios establecidos por Bauer, es usado para la determinación del patrón de susceptibilidad a los agentes antimicrobianos[3, 7, 8, 32].

Los niveles de resistencia en microgramos se determina para siete agentes antimicrobianos, empleandose para ello la técnica de dilución seriada en tubos; el inóculo utilizado es aproximadamente 10^5 bacterias[6].

Para determinar la presencia de factores "R" en estas cepas se utilizan como receptoras una *Escherichia coli* K12 F^- lac^+ trp^+, referida simplemente en el trabajo como K12, la cual fué obtenida por cortesís de Emma Galindo de la Sección de Enterobacterias del Hospital Infantil de México.

Esta cepa es sensible por los dos métodos utilizados en el estudio a los agentes antimicrobianos y su sensibilidad fué estable durante todo el estudio a pesar de subcultivos repetidos. Igualmente es usada como cepa receptora una *Escherichia coli* AM20 F^- nal^r 100g/ml lac^+ trp^+, la cual fué obtenida de Sidney Cohen del Departamento de Microbiología del Michael Reese Hospital and Medicine Center de Chicago. Esta cepa se comportó en forma idéntica a la *Escherichia coli* K12 a excepción del desarrollo de resistencia cromosomal al ácido nalidíxico. Una tercera cepa receptora, *Shigella* 62, aislada del paciente P.M. que inicialmente era sensible a la ampicilina, se conjuga con una cepa donante, *Pseudomona* 5 resistente a la ampicilina, aislada posteriormente de las heces de ese mismo paciente.

Desde estas cepas receptoras los determinantes de resistencia fueron nuevamente transferidas a cepas *E. coli* AM20 $azide^r$ y *Salmonella enteritidis ser Dublin* 707 nal^r, las cuales actuaron como receptoras.

La metodología para demostrar transferencia de resistencia es, con algunas variantes, la descrita por Williams Smith & Shiela Halls[48]. Un tubo conteniendo 10ml de caldo cerebro corazón es inoculado con 0.02ml de un cultivo en caldo cerebro corazón de 24 horas de la cepa donante y 0.1ml de un cultivo similar de la cepa receptora. La mezcla es incubada a 35° - 37°C por 24 horas y luego centrifugada. El sedimento es inoculado mediante un hisopo en un medio de selección constituído por Mac Conkey en placa de petri, al cual, en el caso de ser la receptora una *E. coli* K12 o la *Shigella* 62, se le ha incorporado

ampicilina en concentraciones de 20g/ml. Si la receptora es una *E. coli* AM20 azide^r al medio, además de la ampicilina, le es incorporado sodium azide en concentraciones de 0.004 M. De ser la receptora utilizada una *E. coli* AM20 nal^r o una *S. enteritidis ser Dublin* 707 nal^r un segundo antimicrobiano, ácido nalidíxico en concentraciones de 20g/ml es incorporado al medio, ello facilita la evidencia de la transferencia por cuanto en estas condiciones unicamente crece en las placas la cepa receptora que ha adquirido resistencia del donante. Placas sin antibioticos son utilizadas como controles. Después de 18–24 horas de incubación a 35° - 37°C las colonias de la receptora pueden distinguirse facilmente de las colonias de *Shigella*, en base a la fermentación de la lactosa por la primera y el fracaso de hacerlo la segunda. Para mayor seguridad en el establecimiento de la identificación de la receptora después de la conjugación, a cada cepa le son estudiadas las reacciones en TSI, producción de gas, indol, mótilidad y acetato de sodio.

A fin de verificar la transferencia de resistencia a la cepa receptora le es practicado, después de la conjugación, un estudio de susceptibilidad a los 14 agentes antimicrobianos utilizados en el trabajo.

Los métodos utilizados para establecer criterios de sensibilidad o resistencia son idénticos a los descritos en el estudio inicial de susceptibilidad de las shigelas y de las cepas receptoras antes de la conjugación.

Resultados

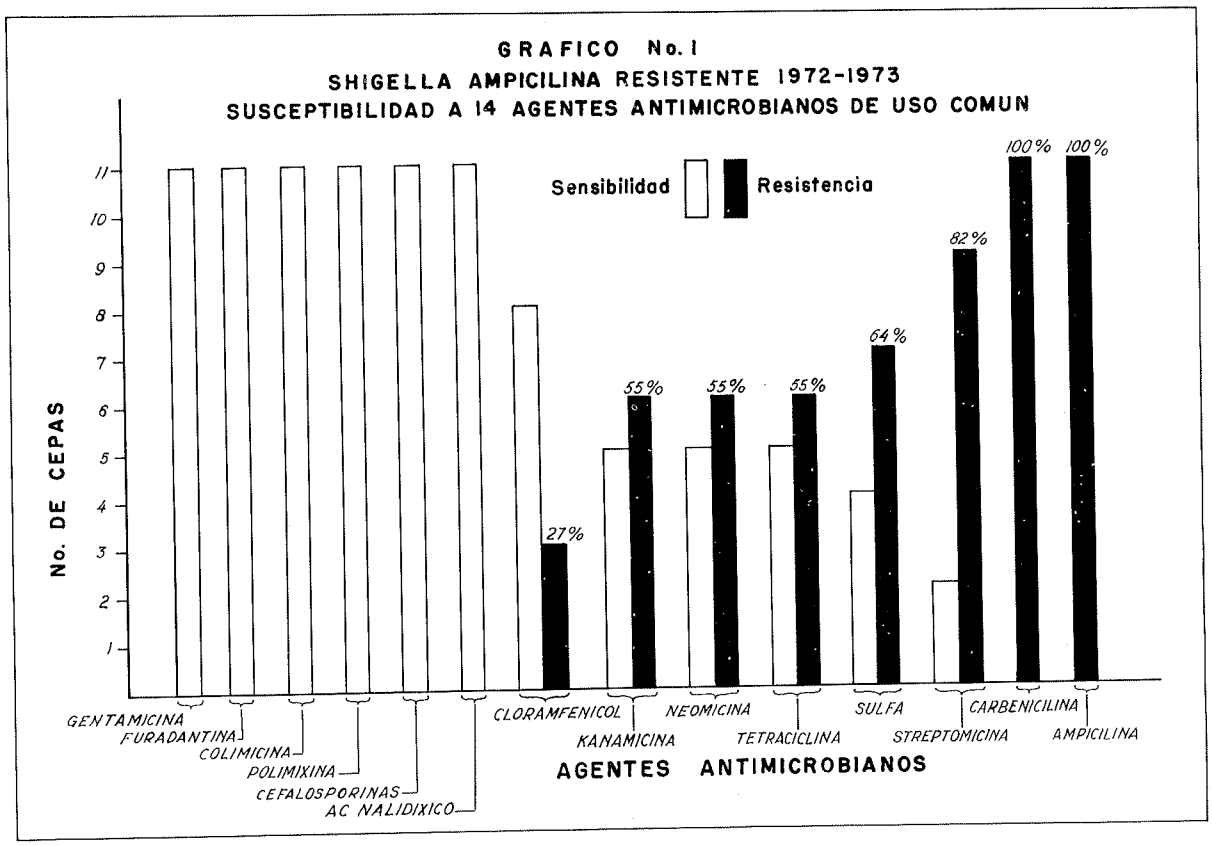
En la tabla 1 puede apreciarse la distribución por grupo y serotipo de once cepas de shigelas resistentes a la ampicilina. Diez pertenecen al grupo B (91%) y dentro de este grupo, cinco de las cepas son del serotipo B_2 (45.45%) y cuatro del serotipo B_1 (36.36%). De las dos restantes una pertenece al serotipo B_4 y la otra al grupo D.

Tabla 1

Shigella ampicilina resistente 1972–1973
distribucion por grupo y serotipo

Grupo	Serotipo	Nº de cepas	Porcentaje
B	B_2	5	45.45
B	B_1	4	36.36
B	B_4	1	9.09
D		1	9.09
Total		11	99.99

El gráfico 1 muestra los patrones de resistencia y sensibilidad a 14 agentes antimicrobianos, incluyendo ampicilina. Todas las once cepas que adquieren resistencia a la ampicilina lo hacen simultáneamente a la carbenicilina. En dos de las cepas estudiadas, este es el único determinante de resistencia existente. En las otras cepas están presentes determinantes de resisten-



cia para otros antimicrobianos. En las cepas se alcanza el 82% de resistencia para streptomicina y 64% para sulfa. Para tetraciclina y kanamicina-neomicina el porcentaje de resistencia es del 55% y cloramfenicol 27%.

No se evidencia aún resistencia para los siguientes agentes gentamicina, furadantina, ácido nalidíxico, colimicina, polimixina-B y cefalosporinas.

En la tabla 2 se muestra la distribución de resistencia múltiple en las once cepas de shigelas resistentes a la ampicilina. Dos de las cepas son resistentes a dos agentes antimicrobianos. Las nueve restantes muestran patrones de resistencia múltiple complejos que le confieren resistencia, desde cinco a ocho antimicrobianos.

Tabla 2

Shigella ampicilina resistente 1972–1973
distribucion de resistencia multiple

Agentes antimicrobianos	Nº de cepas
0	–
1	–
2	2
3	–
4	–
5	4
6	2
7	1
8	2

La tabla 3 permite apreciar los patrones de resistencia múltiple de las cepas estudiadas, observándose la existencia de siete diferentes patrones de resistencia. Dos presentan el patrón de resistencia ampicilina-carbenicilina. En otras dos cepas el determinante de resistencia ampicilina-carbenicilina se acompaña de otros determinantes de resistencia, para constituir un patrón de resistencia múltiple muy complejo constituído por ampicilina-carbenicilina-cloramfenicol-kanamicina-neomicina-streptomicina-sulfa tetraciclina. En otra cepa se observa el patrón de resistencia ampicilina-carbenicilina-kanamicina-neomicina-streptomicina-sulfa-tetraciclina.

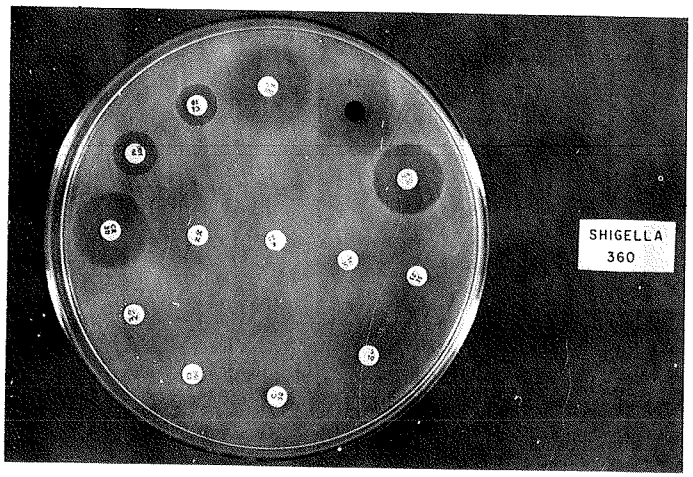
Las once cepas son investigadas por la presencia de factores "R" y en todas ellas se logra la transferencia total de los determinantes de resistencia a las cepas receptoras. En las dos cepas que muestran resistencia solo para ampicilina-carbenicilina los genes r que determinan tal resistencia forman parte de un factor "R", cuyo único determinante de resistencia es el mencionado. En los nueve restantes este determinante al parecer se ha integrado con los determinantes de resistencia de otros agentes antimicrobianos para constituir factores "R" más complejos. Los tipos de factores "R" presentes en las cepas estudiadas y los patrones de resistencia que adquieren las cepas receptoras, *E. coli* K12 F^- lac^+ trp^+ y *E. coli* AM20 F^- nal^r lac^+ trp^+, después de la conjugación pueden evidenciarse en la tabla 4, la movilización del determinante de resistencia para ampicilina-carbenicilina se logra desde estas cepas receptoras, ahora donantes, hacia nuevas receptoras, *S. enteritidis ser Dublin* nal^r 707 y *E. coli* $azide^r$.

Tabla 3

Shigella ampicilina resistente 1972–1973
patrones de resistencia multiple

Tipo de Patron	Nº de cepas
am–cb	2
am–cb–st–su–te	2
am–cb–kn–nm–st	2
am–cb–cm–st–su–te	1
am–cb–nm–st–su	1
am–cb–kn–nm–st–su–te	1
am–cb–cm–kn–nm–st–su–te	2

am = ampicilina; cb = carbenicilina; cm = cloramfenicol; kn = kanamicina; nm = neomicina; st = streptomicina; su = sulfa; te = tetraciclina



Las figuras 1, 2 y 3 ilustran la transferencia de resistencia para ampicilina-carbenicilina-cloramfenicol-kanamicina-neomicina - streptomicina - sulfa - tetraciclina presente en la *Shigella* serótipo B_4 nº 360 a la *E. coli* AM20 F^- nal^r lac^+ trp^+.

Fig. 1 — Muestra el patrón de sensibilidad y resistencia de la *Shigella* 360.

Tabla 4

Shigella ampicilina resistente 1972–1973
resistencia transferible por conjugacion

Donante	Patrones de resistencia multiple	Nº de cepas	Patrones de resistencia multiple transferidos a cepas receptoras*	Nº de cepas
S. flexneri ser B_2 (5 cepas)	am–cb–kn–nm–st–su–te	1	am–cb–kn–nm–st–su–te	1
	am–cb–cm–st–su–te	1	am–cb–cm–st–su–te	1
	am–cb–st–su–te	1	am–cb–st–su–te	1
	am–cb–kn–nm–st	2	am–cb–kn–nm–st	2
S. flexneri ser B_1 (4 cepas)	am–cb–cm–kn–nm–st–su–te	1	am–cb–cm–kn–nm–st–su–te	1
	am–cb–kn–nm–st–su	1	am–cb–kn–nm–st–su	1
	am–cb	2	am–cb	2
S. flexneri ser B_4 (1 cepa)	am–cb–cm–kn–nm–st–su–te	1	am–cb–cm–kn–nm–st–su–te	1
S. sonnei (1 cepa)	am–cb–st–su–te	1	am–cb–st–su–te	1

* *E. coli* K12 F^- lac^+ trp^+ y *E. coli* AM20 F^- nal^r lac^+ trp^+

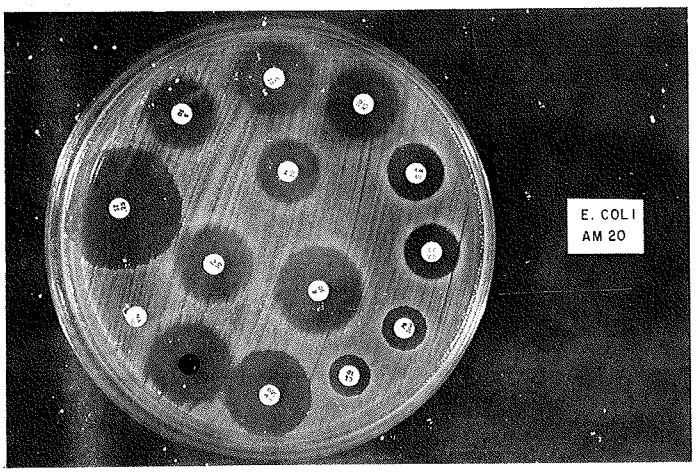


Fig. 2 – Muestra el patrón de susceptibilidad de la cepa *E. coli* AM20 nal^r antes de ser conjugada con la *Shigella* 360.

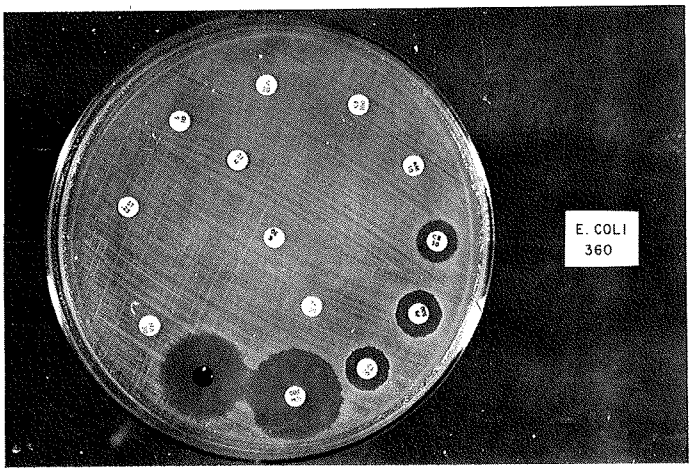


Fig. 3 – Muestra el patrón de sensibilidad y resistencia que adquiere la cepa *E. coli* AM20 nal^r despues de ser conjugada con la *Shigella* 360.

Las tablas 5 y 6 muestran los niveles de resistencia en μg/ml. para los agentes antimicrobianos a los cuales se evidencia resistencia en el estudio. Igualmente muestra los niveles de resistencia adquiridos por la cepa receptora, para estos agentes, después de la conjugación. Es evidente que los niveles de resistencia conferidos por estos determinantes r, tanto en la cepa donante como en la receptora, son por lo general elevados y para varios agentes antimicrobianos, incluyendo ampicilina-carbenicilina se alcanzan niveles superiores a los 1000 μg/ml. En este sentido son ilamativos los resultados obtenidos para agentes como sulfa, cloramfenicol, kanamicina, ampicilina y carbenicilina. Los niveles de resistencia adquiridos por la cepa receptora son en su mayoría de la misma magnitud a los existentes en la cepa donante.

Mención especial merecen, la *Shigella* serotipo $B_2$8031 la cual posee el patrón de resistencia sulfa-streptomicina-tetraciclina-cloramfenicol-ampicilina--carbenicilina aislada del paciente P.M. al 5to día del tratamiento con ampicilina y la *Shigella* serotipo B_2 62 con un patrón de resistencia similar al descrito en la cepa 8031, a excepción de la ausencia de resistencia ampicilina-carbenicilina, aislada del mismo paciente antes de iniciar el tratamiento con ampicilina. Los determinantes de resistencia a la ampicilina, presentes en miembros de la familia Enterobacteriaceae y Pseudomonadaceae que forman parte de la flora intestinal de este paciente, pudieron ser transferidos in-vitro por conjugación a la *Shigella* serotipo B_2 62.

Tabla 5

Shigella ampicilina resistente 1972–1973
Niveles de resistencia en μg/ml conferidos por factores "R" en la cepa donante y la cepa receptora

Agente antimicrobiano	μg/ml	Donante	Receptora
ampicilina	1000	6	4
	500	5	5
	250		2
	125		
	62.5		
	31.2		
	15.6		
	7.8		
	3.9		
	c		
carbenicilina	1000	11	11
	500		
	250		
	125		
	62.5		
	31.2		
	15.6		
	7.8		
	3.9		
	c		
sulfa	1000	11	11
	500		
	250		
	125		
	62.5		
	31.2		
	15.6		
	7.8		
	3.9		
	c		
cloramfenicol	1000	3	3
	500		
	250		
	125		
	62.5		
	31.2		
	15.6		
	7.8		
	3.9		
	c		

Nota: inóculo utilizado 10^5 bacterias por ml.

Tabla 6

Shigella ampicilina resistente 1972–1973
Niveles de resistencia en μg/ml conferidos por factores "R" en la cepa donante y la cepa receptora

Agente antimicrobiano	μg/ml	Donante	Receptora
kanamicina	1000	6	6
	500		
	250		
	125		
	62.5		
	31.2		
	15.6		
	7.8		
	3.9		
	c		
streptomicina	1000	5	
	500	2	3
	250	2	4
	125		2
	62.5		
	31.2		
	15.6		
	7.8		
	3.9		
	c		
tetraciclina	1000		
	500		3
	250	1	3
	125	5	
	62.5		
	31.2		
	15.6		
	7.8		
	3.9		
	c		

Nota: inóculo utilizado 10^5 bacterias por ml.

Discussion

Los resultados presentados al igual que los obtenidos en otros estudios muestran que en nuestro medio, la resistencia transferible por conjugación es frecuente en *Shigella*[40]. Muestran además que la resistencia que ésta entidad bacteriana adquiere contra antimicrobianos introducidos recientemente, asume las características de la resistencia mediada por elementos genéticos extracromosómicos conocidos como factores "R".

En 1969, Prieto llamó la atención sobre el hecho de que los patrones de resistencia múltiple más complejos, presentes en las shigelas, han aparecido en cepas del grupo B (*Sh. flexneri*) y que la resistencia para kanamicina-neomicina observada en el 24% de las shigelas aisladas en la localidad, era exclusividad de los serotipos de este grupo[40]. Ambos aspectos continúan teniendo vigencia en la actualidad. Esta observación adquiere aún mayor validez ya que el determinante de resistencia para ampicilina-carbenicilina, reportado en este trabajo, a excepción de una cepa del grupo D, también ha hecho su aparición en cepas pertenecientes al grupo B y al igual que la resistencia adquirida para kanamicina-neomicina, asume la modalidad de estar presente en varios de los serotipos que integran este grupo. Esto adquiere importancia práctica por cuanto son precisamente las cepas de estos grupos (74%) y (20%), las que prevalecen entre nuestras shigelas.

Aún cuando la resistencia adquirida para ampicilina-carbenicilina puede constituir en algunas shigelas una entidad episomal cuyo único determinante de resistencia es el aludido, en la mayoría (82%) este episoma, ya sea conservando su individualidad genética (estado hetero-R) a lo que parece ser más probable, incorporando sus determinantes de resistencia a los que integran factores "R" complejos que median multirresistencia, dotan a estas shigelas de patrones de resistencia complejos, en dos de ellas se constituye así un patrón de resistencia, sulfa-streptomicina-tetraciclina-cloramfenicol-kanamicina-neomicina-ampicilina-carbenicilina. Este patrón solamente ha sido descrito hasta el presente en una cepa aislada en Perú en 1970 y estudiada por Carpenter, en el Dysentery Reference Laboratory de Londres[24], y en otra reportada en Japón por Tanaka & col. en 1966, en un estudio realizado sobre 9.635 cepas[53]. Este patrón adquiere precisamente las características de uno descrito por Prieto & col. en *S. enteritidis ser Saintpaul* y *bioser Java*, serotipos que fueron responsables de dos brotes epidémicos ocurridos en la localidad en el transcurso de los años 1968–1972.

En *Shigella* el determinante de resistencia para ampicilina, al igual que en *Salmonella* y a diferencia de otras entidades bacterianas, parece ser el mismo que media resistencia para carbenicilina, debido a que en este estudio como en otros, no se logra evidenciar segregación de los genes que determinan esa resistencia[41].

En relación al paciente P.M., es tentador especular que la adquisición in-vivo de resistencia a la ampicilina se efectuara en el tracto intestinal al concurrir factores importantes para que se adquiera tal tipo de resistencia, como son la existencia de flora intestinal portadora de factores "R" con determinantes de resistencia para dicho antimicrobiano y la presión selectiva impuesta por su uso[4, 25, 26, 49, 54]. Evidencias de que ello ocurre in-vitro fueron obtenidas en este caso.

Las bases bioquímicas que median la resistencia a ampicilina son de naturaleza enzimática[11, 24]. En la actualidad en nuestro laboratorio se realizan trabajos para la caracterización de una beta lactamasa producida por estas cepas, que resulta ser la responsable de su resistencia.

Los niveles de resistencia obtenidos para ampicilina-carbenicilina al igual que los observados para la mayoría de los agentes antimicrobianos a los cuales se adquiere resistencia por este mecanismo, son con frecuencia superiores a los 1000 μg/ml. Resulta también de interés el hecho que dichas cepas puedan transferir en la misma magnitud tal resistencia a cepas receptoras sensibles. En clínica estos factores "R" que median altos niveles de resistencia constituyen un problema serio, ya que interfieren con el éxito del tratamiento, aún cuando la dosis utilizada del antimicrobiano en cuestión sea la más elevada que el organismo humano sea capaz de tolerar. La aparición de esta resistencia en estas cepas, constituye otra justificación para hacer obligatorio en nuestro medio el uso rutinario de pruebas de sensibilidad y resistencia antes de emprender una terapia en el tratamiento de la shigelosis, si se aspira que ella resulte efectiva. Ya que la mayoría de estas cepas resistentes a la ampicilina son también resistentes a los antimicrobianos considerados por lo general de utilidad en el tratamiento de tal entidad clínica (tetraciclina, cloramfenicol, kanamicina y sulfa) es de esperarse que el problema del tratamiento antimicrobiano de la shigelosis llegara a ser mucho más dificil de lo que ha sido en el pasado. Además es razonable esperar que a medida que el uso de ampicilina se incremente en este país, la shigelosis por *Shigella* ampicilina resistente, aumentará en frecuencia e importancia.

Al igual que ha sido demostrado en otros paises, en nuestro medio la rápida aparición de resistencia, especialmente del tipo descrito en estas cepas, está intímamente ligado al uso indiscriminado que se le ha dado y continúa dandosele a los antimicrobianos, tanto en el medio hospitalario como en la comunidad.

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Transferable gentamicin-resistance among enterobacteria

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Summary

Out of 62 cultures of *Salmonella typhimurium, Escherichia coli, Klebsiella-Enterobacter* and *Proteus mirabilis* that have been studied, 56 were able to transfer gentamicin-resistance into *E. coli* K12 during mixed cultivation. Although the R-type of gentamicin-plasmids were not uniform with respect to different donor species, all of them were Fi negative and resistant to kanamycin and tobramycin in addition to gentamicin. The selection and spreading of gentamicin resistance, among enterobacteria, might result from the extensive use of the antibiotic in treatment of infantile enteritis in Recife, Brazil.

Resumo

Resistência transmissível à gentamicina nas enterobactérias

Entre 62 culturas de *Salmonella typhimurium, Escherichia coli, Klebsiella-Enterobacter* e *Proteus mirabilis* 56 transferiram a gentamicina-resistência para a *E. coli* K12 durante a conjugação. Embora as várias espécies doadoras tenham fornecido plasmídios com diferentes modelos R, todos os plasmídios selecionados pela gentamicina apresentaram-se Fi negativos e resistentes também à kanamicina e tobramicina. Atribuiu-se a seleção e disseminação da gentamicina-resistência, entre as enterobactérias, ao largo emprego do antibiótico no tratamento das enterites infantis, no Recife.

Introduction

Over the past four years an increasing number of infantile gastroenteritis outbreaks in Recife, Brazil, have been linked to a single multiresistant phage type of *Salmonella typhimurium.*

Illness was characterized by severity and there was no response to the standard regimen of treatment. To control the infection, gentamicin was administered orally in daily dosage of 20–30mg/Kg body weight as well as by intramuscular injection of 2–4mg/Kg body weight. Simultaneous oral and intramuscular administration was maintained for 5–10 days. After two years, as a result of this generalized therapeutic policy, over 60% of the clinical *S. typhimurium* isolates were resistant to the drug. Presently some of the other gram-negative species are also starting to present resistance to gentamicin.

This paper reports the characteristics of gentamicin resistance among cultures of enteric bacilli recovered from clinical sources in Recife.

Material and Methods

Organisms – Twenty strains each of *S. typhimurium, Escherichia coli, Klebsiella-Enterobacter*, and two of *Proteus mirabilis* were selected. Cultures were isolated in 1974 in Recife, Brazil. As receptor *E. coli* K12 F^- Rif^r was used; as intermediate donors, *E. coli* K12 Δ or *E. coli* K12 X were used, and for Fi testing *E. coli* K12 Hfr H and the male specific phage μ^2. These microorganisms were kindly supplied by Dr. E.S. Anderson from the Enteric Reference Laboratory of London.

Susceptibility tests – These tests were carried out by an agar plate dilution technique employing MacConkey medium (BBL) for the antibiotics, and Muller-Hinton agar (BBL) for sulphamethoxazole. Cultures to be tested were grown overnight in brain heart infusion (BHI) and diluted in saline so to contain about $1–5 \times 10^6$ colony forming units per ml. A loopfull (0.01ml) of diluted culture was

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spotted onto the surface of the drug agar medium. A culture was considered resistant if it was able to grow in an amount of drug 8-fold above the minimal inhibitory concentration (MIC) of the standard suscetible *E. coli* K12 R^- strain. The abbreviations and MIC of the drugs studied (in μg/ml) was as follows: ampicillin (A) 5; chloramphenicol (C) 5; kanamycin (K) 2.5; paromomycin (P) 10; streptomycin (S) 5; sulphamethoxazole (Su) 40; tetracycline (T) 5; gentamicin (G) 1; and tobramycin (To) 0.5 μg/ml.

Resistance transfer — Cultures to be crossed were grown overnight in BHI and mixed with, donor and receptor in a proportion of 0.1:0.9ml. Afterwards, 4ml of fresh BHI were added to the mixture. In order to detect eventual non self-transferable R-determinants, a mobilization test, involving Δ and X resistance transfer factors, was also performed[1]. Mating mixtures were incubated at 37°C for 18 hours and spotted on appropriate selective medium for the isolation of drug resistant recombinants. Frequencies of transfer were calculated in terms of the proportion of the recipient population that had received the R factor under study. Linkage of the gentamicin character with other R-determinants was studied by testing for resistance in each of ten colonies ramdomly taken from a gentamicin agar plate.

Fertility inhibition (Fi) — To test for the Fi character of different plasmids, they were re-transferred to *E. coli* Hfr H which was then tested for sensitivity to the male specific phage μ[2].

Results

The spectrum of drug-resistance shown by wild cultures is presented in Table 1. Level of resistance to gentamicin ranged between 8 and 64 μg/ml but the great majority of the strains (90,3 per cent) presented levels of 16–32 μg/ml (Table 2).

Table 1

Resistance pattern of wild strains

R-type	*S. typhimurium*	*E. coli*	*K-Enterobacter*	*P. mirabilis*
A C G K P S Su T To	12	16	5	2
A C G K P S Su To	8		2	
A C G K P Su T To		4	7	
A G K P S Su To			6	

Table 2

Levels of G-resistance among wild cultures

Organisms	Gentamicin (μg/ml)			
	8	16	32	64
S. typhimurium		8	10	2
E. coli		17	3	
K-Enterobacter	2	16	2	
P. mirabilis	2			

Table 3

Transfer and linkage of the plasmid Gk isolated from *Enterobacteriaceae*

Species*	Plasmid R-type		Cultures
S. typhimurium 20 (20)	ASSu	Gk	14
	A	Gk	10
	ASSuKp	Gk	6
	AKp	Gk	6
	CTKp	Gk	2
E. coli 20 (16)	GK		6
	ASSu	Gk	2
	SSuT	Gk	2
	ACKpSSuT	Gk	4
	ACKpSu	Gk	4
	Kp	Gk	4
	CKpSSuT	Gk	4
K-Enterobacter 20 (18)	AKpSSu	Gk	10
	AKp	Gk	16
	Kp	Gk	6
	ACKpSSuT	Gk	2
	A	Gk	2
	KpSSu	Gk	2
P. mirabilis 2 (2)	ACKpSSu	Gk	2

* Number within parenthesis indicates cultures which transferred the plasmid Gk.

The ability of the enterobacteria to transfer gentamicin-resistance have shown a high efficiency of transmission. However, the R-type of gentamicin plasmids were not uniform with respect to the different donor species involved. Thus, *S. typhimurium* transferred ASSuKGTo more frequently while *E. coli* transferred KGTo and *Klebsiella* AKPGTo (Table 3). Despite these minor differences in R-type, all of the G-plasmids isolated were Fi negative, belonged to group Gk and were re-transferred to *E. coli* Hfr H as a single linkage group. Furthermore, they were transferred at about $1 \times 10^{-5} - 5 \times 10^{-2}$ in overnight crosses to *E. coli* K12 and independent of donor species.

Discussion

Present results have shown that the excess use of gentamicin will result in the development of strains resistant to it and other aminoglycoside antibiotics.

Although it might be difficult to establish the origin of plasmids governing gentamicin resistance, there is a high probability that they were first selected in multiresistant *S. typhimurium* as a response to over generous use of the antibiotic in pediatric units. Indeed, gentamicin resistance was former detected in that species and the different cultures of *S. typhimurium* which have been studied probably represent only one strain showing a high ability to spread R factors. Therefore, multiresistant *S. typhimurium* seems to be, at least among the enterobacteria, the source of gentamicin plasmids in Recife.

Transferable resistance to gentamicin has been studied in detail by French researches[3,4,5] who were able to classify some of the plasmids involved in the inactivation of the drug. The first of those described was the plasmid Gk[3] which determines the synthesis of an adenylytransferase[2]. This enzyme adenylates kanamycin and tobramycin in addition to gentamicin. In the present investigation, all the plasmids isolated belonged to type Gk. However, they were neither uniform with respect to their linkages with other resistance determinants, nor were their R-types similar to those found in Paris[4]. Despite these minor variances all of them, like the French plasmids, were Fi negative, belonged to Class 1, that is, the resistance determinants and the transfer factor were transmitted as a single linkage group, and manifested a high efficacy of mobilization into *E. coli* K12.

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