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Myocarditis: an expected health hazard associated with water resources contaminated with Coxsackie viruses type B

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Enteroviruses, especially Coxsackie B viruses (CBVs), are responsible for approximately 50% of cases of viral myocarditis. In the present study, serum samples (160) were collected from acute myocarditis patients at different age groups and 104 samples of the same age groups as a control. Cholesterol, LDH, CPK, and GOT were measured for all serum samples (264). Also, to study the source of virus transmission, 72 water and 72 wastewater samples were collected from water and wastewater treatment plants at intakes and outlets. Water and wastewater samples were concentrated by filtration through Zeta-plus filter cartridges and reconcentrated by the PEG-6000 precipitation method. Serum, water, and wastewater samples were inoculated in BGM cells for three successive passages. RT – PCR with enterovirus primers was carried out directly for serum samples and for 1st and 3rd cell culture passages. The positive samples were used for neutralization assay using anti-CBV sera pool to determine the CBV followed by neutralization with separate antisera. The results showed that 50 (31.25%) serum samples from acute myocarditis patients and two (1.4%) samples from the controls were positive for enterovirus RT – PCR. For water and wastewater samples enteroviruses were present in 63.8% and 8.3% for intake and outlet of water treatment plants and, 66.6% and 47.2% for intake and outlet of wastewater treatment plants, respectively. The level of CBV serotypes was varied where CBV3 was dominant for all age groups of myocarditis patients and CBV2 and CBV5 were also detected while CBV2 was the main CBV in water samples and CBV2, 3 and 5 were detected in wastewater samples. The integration of cell culture-PCR reduces the time required for virus detection and enhances the sensitivity of the test.

Keywords: Myocarditis; myocardial infarction; water; wastewater; Coxsackie B viruses.

Introduction

The presence of enteroviruses in the environment is a public health hazard even when few viral particles are present (Schiff *et al*. 1984; Rose and Gerba 1991). Vehicles for virus dissemination into the environment are stools, skin lesions, nasal and bronchial secretions, and tears. Contaminated water and wastewater are the main sources of enteroviruses including coxsackieviruses type B which may be transmitted to humans. Epidemiological studies are difficult, since those infected by the virus may act as carriers but show no symptoms. The disease may become apparent only after another person has become infected, which may occur at a distance from the original source (WHO 1979). Methods of virus spread are oral and respiratory, either from direct source or indirectly through fecal pollution of food and families.

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Hosts that secrete enteroviruses, which cause human infections whether subclinical or clinical, are acutely infected patients and/or animals. The wide spread of enteroviruses in the environment represents a public health hazard especially in the early years of life. It is surprising that few reported illnesses were documented as enteroviral. An example to the point is viral myocarditis affecting neonates, children, and adults (Martin *et al*. 1994 and Bowles and Towbin 2000).

Myocarditis, the acquired form of inflammatory heart muscle disease, is manifested as an acute and chronic condition (Sole and Lui 1993). In children, acute onset of congestive heart failure is common and echocardiography is consistent with dilated cardiopathy. In adults, chronic disease is more common, presenting an idiopathic dilated cardiomyopathy. The most common etiology for myocarditis, particularly in children, is viral (Schonian *et al*. 1995). The enteroviruses, particularly coxsackievirus B3 and B4, have been considered major etiologic agents (Sole and Lui 1993). In addition, echoviruses, coxsackieviruses A, adenovirus and cytomegalovirus have also been implicated (Martin *et al*. 1994; Schonian *et al*. 1995). Pauschinger *et al*. (1999) reported that adenoviruses and enteroviruses are equally important causes of left ventricular dysfunction in adults. These findings were confirmed by some studies where concomitant increases in antibodies to coxsackieviruses B were seen at diagnosis of myocardial infection. Another cardiac disease is myocardial infarction (MI), associated with rising antibodies against coxsackieviruses type B, suggesting the association of infections also due to this virus group with MI (Wood *et al*. 1975; Nicholls and Thomas 1977; Nikoskelainen *et al*. 1983; and Roivainen *et al*. 1998). In this study, the role of coxsackieviruses type B in adult cardiac disease and their dissemination via wastewater and water resources was evaluated.

Materials and methods

Cardiac patients

Serum samples were collected from 115 men and 45 women between 25 and 70 years of age. They were selected randomly from mostly first time admissions to the Cardiac Care Unit (CCU) of El-Sahel Teaching Hospital in Cairo. Patients were clinically diagnosed as acute myocarditis. Serum cholesterol, Lactate dehydrogenase (LDH), creatine phosphate kinase (CPK) and glutamic-oxaloacetic transaminase (AST) were measured according to the manufacturer (Randox Laboratories Ltd., USA).

Control samples (104 serum samples) from healthy adults volunteering for blood donation were chosen at random to match age and sex of the cardiac patients covered by this study and treated identically.

Environmental samples

Water and wastewater samples (72 water and 72 wastewater) were collected from three water treatment plants (El-Giza, El-Maadi and Mostorod) and three wastewater treatment plants (Zeinin, El-Berka and Balaks) in greater Cairo. Each water sample amounted to 40 l, whereas for wastewater 5 l were collected. Samples were picked up monthly (from November, 1999 to October, 2000) for each treatment plant from the intake and outlet of the plants. The chlorinated samples were dechlorinated with 5 mg/l sodium thiosulphate.

Virus concentration

Water samples were concentrated by adsorption on Zeta plus cartridge (1MDS Virosorb, 10 inch length). The adsorbed viruses were eluted in 1 l of 3% beef extract-0.05 M glycine

solution, pH 9.5 as described by Goyal and Gerba (1983). Raw wastewater samples were left to precipitate the sludge, which was used for direct elution of the viruses in 3% beef extract–0.05 M glycine solution, pH 9.5 (50 g/200 ml eluent) with stirring for 30 min. The supernatant and the treated effluent samples were filtered through nitrocellulose membrane, 0.45 μm pore size and the adsorbed viruses were eluted by the same eluent. Eluate of each sample was neutralized to pH 7.0 and viruses were reconcentrated by PEG 6000 precipitation method (le Guyader *et al*. 1995). PEG 6000 [50% (w/v) solution in phosphate buffered saline] was added in a final 10% concentration to the eluates. The pH was adjusted to 7.4 and the mixture was stirred for 2 h at 4°C and centrifuged for 30 min at 10,000 × g at 4°C. The pellet was suspended in 5 ml of sterile deionized water. The concentrated samples were extracted twice with freon (1,1,2-trichlorotrifluoroethane, Merk) and 50 μl of 100X antibiotic-antimycotic mixture (10,000 U penicillin G sodium, 10,000 μg streptomycin sulphate and 25 μg amphotericin B, GIBCO-BRL) was added per each 5 ml concentrated sample.

Cell culture

Buffalo green monkey (BGM) kidney cells were used for three successive passages of the serum (diluted 1 : 10 in 0.01% SDS in phosphate-buffered saline, pH 7.4) as well as the concentrated water and wastewater samples. Cells were grown in Dulbecco's minimum essential medium with Glutamax-II (GIBCO–BRL). The medium was supplemented by 10% foetal bovine serum and 1% antibiotic-antimycotic mixture. Viruses in samples that induced cytopathic effect (CPE) in BGM cells for the three successive passages were harvested by freezing and thawing three times.

Oligonucleotides

Oligonucleotide primers, E1 and E2 were purchased from Research Genetics (UK). Primers were derived from the highly conserved sequences in the nontranslated region of the enterovirus genome (Chapman *et al*. 1990). The sense one, E2 (5′-TCC GCC CCC TGA ATG-3′, nt 446 to 460) and antisense E1 primer (5′-CAC CGG ATG GCC AAT CCA-3′, nt 640 to 623), were diluted in TE buffer and adjusted to approximately 5 μM, and stored at − 20°C.

RNA extraction

Viral RNA was extracted directly from serum samples and cell culture lysates of CPE-induced serum, water and wastewater samples at both 1st and 3rd passages in BGM cells by the cetyltrimethylammonium bromide (CTAB) method, described by Jiang *et al*. (1992). Samples were treated with an equal volume of Freon and after clarification, viruses were concentrated with 8% polyethylene glycol 6000 (Sigma, PEG-6000) in the presence of 0.4 M sodium chloride. After digestion of the pellet with proteinase K (400 μg/ml, Serva Biosystems) in proteinase K buffer (0.1 M Tris-HCl, pH 7.5, 12.5 mM EDTA, 0.15 M NaCl, 1% SDS) for 30 min at 37°C, CTAB and NaCl were added to final concentrations of 1.25% and 0.45 M, respectively. The solution was incubated at 56°C for 30 min and extracted with an equal volume of phenol-chloroform and reextracted with an equal volume of chloroform. The RNA was precipitated in 2.5 volumes of ethanol and resuspended in 20 μl of DEPC-treated water for use in the RT reaction.

cDNA synthesis and PCR

To 5 μl extracted RNA the following reaction mixture was prepared: 2 μl of 2.5 mM each dNTP, 1 μl (0.25 μmol/l) antisense primer E1 and 1 μl (10U) of reverse transcriptase from

Rous-associated virus 2 (RAV-2,TaKaRa Biomedicals, Japan), 4 μl of 5 × RT buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 40 mM $MgCl_2$, 50 mM DTT) and 7 μl DEPC-treated water and 50 μl mineral oil. The mixture was heated at 42°C for 30 min followed by 99°C for 5min, then chilled on ice. To 5 μl of the synthesized cDNA the following were added to reaction volume of 50 μl: 1 μl of 2.5 mM of each dNTP, 1 μl of each sense and antisense primer, 5 μl of 10 × PCR buffer containing 2 mM $MgCl_2$ and 1 μl (5U) TaKaRa Ex Taq DNA polymerase, 36 μl DEPC-treated water. The mixture was covered by 50 μl mineral oil. The PCR mixture was subjected to the following temperature condition, 95°C, 55°C and 72°C for 1 min each for 40 cycles. The PCR product was detected in 2% agarose gel containing 0.5 μg ethidium bromide and visualized under UV light comparing to the 100 bp ladder (GIBCO – BRL).

Neutralization assay

Neutralization test was carried out with coxsackievirus B antisera types 2 to 6, purchased from American Type Culture Collection (ATCC, Cat. no. V029-501-560 to V033-501-560) in BGM cells. The identification procedure was performed in two steps. In the first step, anti-CB sera pool was used and prepared by mixing the separate antisera in 1 : 10 ratio to recognize the CB viruses in the enteroviruses RT – PCR positive samples. The second step was carried out for the CB neutralized samples using separate antisera (Melnick *et al.* 1973).

Results

Incidence of enteroviraemia in acute cardiac patients by RT – PCR

Direct detection of enteroviraemia in patient's sera was achieved. As shown in Table 1 there was a higher incidence in patients aged between 46 – 65, which dropped to one tenth by age $\geqslant$ 66 years. The use of RT – PCR followed by isolation in BGM cells has improved detection of enteroviraemia in patient sera. The detection signals were more evident in the age group $\geqslant$ 66 years, where 15 (25%) were RT – PCR positive for enteroviruses compared to one (1.6%) identified as positive by direct serum test. The enterovirus signals were nearly doubled in patients' age group 46 – 65 and almost so in patients' age group 25 – 45 years. This finding stresses the value of amplifying the virus load in serum by allowing virus replication in BGM cells. It also shows that the functional presence of antiviral activities like antibody-and/or interferon in a serum sample was nullified when the serum was inoculated into cell culture.

Relation between enteroviraemia and blood chemistry changes

The values of CPK, AST and LDH were high, indicative of myocardial injury. Comparison of these changes with cholesterol values which are not very elevated, weights the evidence of an acute infections process rather than an atherosclerosis coronary occlusion etiology among those patients.

Incidence of coxsackie B serotypes in cardiac patients

Coxsackieviruses represent three out of five (60%) of the enteroviraemia in the cardiac patients age group 25 – 45 years (Table 3). There was one coxsackievirus B2 and two coxsackievirus B3 isolates. This relative incidence of coxsackieviraemia in enteroviraemic cardiac patients was different in the age group 46 – 65 years. Only five out of 30 (16.6%) of the viraemia were coxsackie B viruses (2 CoxB2, 2 CoxB3 and 1 CB5). In the older age group of cardiac patients, a relative rise of coxsackieviraemia in the enteroviraemia was noticed. There were six out of 15 (40%) coxsackieviruses (4 CB3 and 2 CB5). Considering the sum of coxsackievirus isolates,

Table 1. Incidence of enterovirus RNA by RT – PCR in different age group of myocarditis patients and normal controls in relation with cholesterol, LDH, CPK and AST

		*Blood chemistry**				*No. of RT – PCR positive for EV*	
Age group (Year)	*Sex M/F*	*Chol*	*LDH*	*CPK*	*AST*	*Direct serum*	*1st cell culture passage*
25 – 45	20/5	206 ± 24.08	1253.4 ± 458.01	628 ± 237.63	22.6 ± 3.91	3 (12%)	5 (20%)
(NC)	(26/3)	197 ± 8.36	157.4 ± 41.51	35.4 ± 18.59	9.75 ± 0.95	0 (0%)	0 (0%)
46 – 65	50/25	247.7 ± 42.01	910.54 ± 306.8	488 ± 230.28	28.77 ± 8.27	12 (16%)	30 (40%)
(NC)	(60/1)	229.56 ± 23.44	175.21 ± 38.62	48.26 ± 15.31	11.75 ± 2.06	0 (0%)	2 (3.2%)
⩾ 66	45/15	246 ± 76.05	999.5 ± 273.26	621.75 ± 132.19	37.25 ± 18.24	1 (1.6%)	15 (25%)
(NC)	(14/0)	225 ± 19.14	187.5 ± 41.93	56.25 ± 14.93	12.25 ± 1.25	0 (0%)	0 (0%)

(NC) Normal controls
M/F Male/Female
*Expressed as mean ± SD
Chol Cholesterol (N.150 – 280 mg/dl)
LDH Lactate dehydrogenase (N.120 – 240 u/l)
CPK Creatine phosphate Kinase (N.10 – 80 u/l)
AST Serum Glutamic-oxaloacetic transaminase (N.up to 12 u/l)
EV Enteroviruses

there was 3 CB2, 8 CB3 and 3 CB5 suggesting a frequent link of B3 serotype to acute cardiac illness than either or both serotypes B2 and B5 in these patients aged 25– ⩾ 66 years.

Silent enteroviremia in healthy subjects

Recovery of an enterovirus from sera drawn from healthy subjects was only achieved in the age group 46–65 years when two out of 61 (3.2%) sera gave an enterovirus RNA signal by RT–PCR (Table 1). At large only two out of 104 (1.9%) samples were positive for enterovirus-RNA in comparison with 50 out of 160 (31.2%) sera from cardiac patients. This difference ($P > 0.05$) is statistically significant.

RT–PCR for water and wastewater samples

RT–PCR was carried out for both first and third passage of the concentrated water and wastewater samples. The results showed that the integration of cell culture-PCR in both first and third passage is the same. Also, there was no difference in enterovirus level of raw Nile water at WTP intakes and the raw sewage where the number of positive samples by RT–PCR for enteroviruses are nearly the same (23 out of 36 for WTPs and 24 out of 36 for WWTPs). This finding showed how the intakes of the WTPs studied are polluted (Table 2). In both El-Maadi and Mostorod drinking water treatment plants, it was observed that the incidence of enteroviruses in the final treated water was more than in intakes.

Incidence of coxsackie B serotypes in cardiac patients, water and wastewater

Coxsackieviruses type B have been detected in water and wastewater by many investigators (Montserrat *et al*. 1994; Wyn-Jones *et al*. 1995). In this study, the coxsackie B viruses were detected by neutralization assay using both anti-CB sera pool and separate sera. The young age group had a higher percentage of CB viruses than the other two groups, where CB viruses were 60% of the positive enterovirus samples. In case of water and wastewater samples the percentage of CB viruses was 14.2% and 33.3% for intake and outlet of water treatment plants, respectively and 33.3%, 29.4% for wastewater treatment plants as shown in Table 3.

Table 2. Enterovirus detection in water and wastewater samples by RT–PCR

	Intake		*Outlet*	
Water and wastewater treatment plant	*No. of samples*	*No. of positive RT–PCR samples*	*No. of samples*	*No. of positive RT–PCR samples*
1-WTPs				
EL-Giza	12	11 (91.6%)	12	3 (25%)
El-Maadi	12	8 (66.6%)	12	9 (75%)
Mostorod	12	4 (33.3%)	12	9 (75%)
Total	36	23 (63.8%)	36	21 (58.3%)
2-WWTPs				
Zenin	12	8 (66.6%)	12	8 (66.6%)
El-Berka	12	6 (50%)	12	3 (25%)
Balaks	12	10 (83.3%)	12	6 (50%)
Total	36	24 (66.6%)	36	17 (47.2%)

Table 3. Percentage of Coxsackieviruses type B in enterovirus positive samples

Sample	*Sample source*	*No of positive EV*	*No of CB*	*% of CB*	*CB serotypes*
Serum	25–45 Y	5	3	60	1CB2, 2CB3
	46–66 Y	30	5	16.6	2CB2, 2CB3, 1CB5
	⩾ 66 Y	15	6	40	4CB3, 2CB5
Water	Intake	23	3	14.2	3CB2
	Outlet	5	1	33.3	CB2
Wastewater	Intake	24	8	33.3	3CB2, CB3, 4CB5
	Outlet	17	5	29.4	3CB2, 2CB5

Y age by year
Cox Coxsackieviruses.

The level of each CB virus serotype differed greatly between the samples studied. CB3 virus was dominant in the serum samples of the three age groups (Fig. 1A) while CB2 showed the highest level in water and wastewater samples (Fig. 1B).

Discussion

Enterovirus infection produces viraemia before the virus localizes in the target organ. The recovery of the virus from blood strongly documents an etiologic association. This is more valid when healthy subjects as controls were examined for an enteroviraemia.

In this study, sera of cardiac patients and of control subjects were examined for an enteroviraemia by RT–PCR. There were stark differences between the incidence of a positive enteroviral RNA signal by RT–PCR. A total of 16 out of 160 (10%) of cardiac patients sera compared to two out of 104 (1.9%) control subject sera were positive for an enteroviraemia. This difference is statistically significant ($P > 0.05$). This difference was more evident when BGM cell cultures were used to isolate the enterovirus from the sera by inoculation for a first passage. This step was successful in amplifying the virus load in serum by allowing the free virus to replicate, irrespective of the effect of antiviral serum activity. Viral replication increases viral RNA concentration. We strongly recommend this step for serum viral load amplification, although it means awaiting the harvest of an experimentally virus-infected cell culture after 2–3 days and an added cost of maintaining an enterovirus-sensitive cell culture in the laboratory. Also, an association of coxsackievirus B-3 with acute cardiac illness was more frequent than coxsackievirus B-2 and coxsackievirus B-5 in this descending order. In an ongoing study, coxsackievirus serotype B-2 was detected frequently in water as environmental source and Coxsackieviruses B-2, B-3 and B-5 were isolated from wastewater. Municipal water containing an enterovirus should be considered as an active vehicle of transmission and dissemination. All age groups consume water; therefore they are liable to infection by one or more of coxsackievirus serotypes B-2, B-3 and B-5. However, considering our findings, community-acquired coxsackievirus B-2 infection could occur as early as the starting age of drinking municipal water. Yet when the cardiac patients were specifically screened for enteroviraemia coxsackieviruses B-3 and B-5 were more prominent than B-2. These findings suggest that early exposure to coxsackievirus B-2 in the environment, mostly through drinking municipal water, reduced the prominence of serotype B-2 as etiologic cause of adult acute cardiac illness. Adult

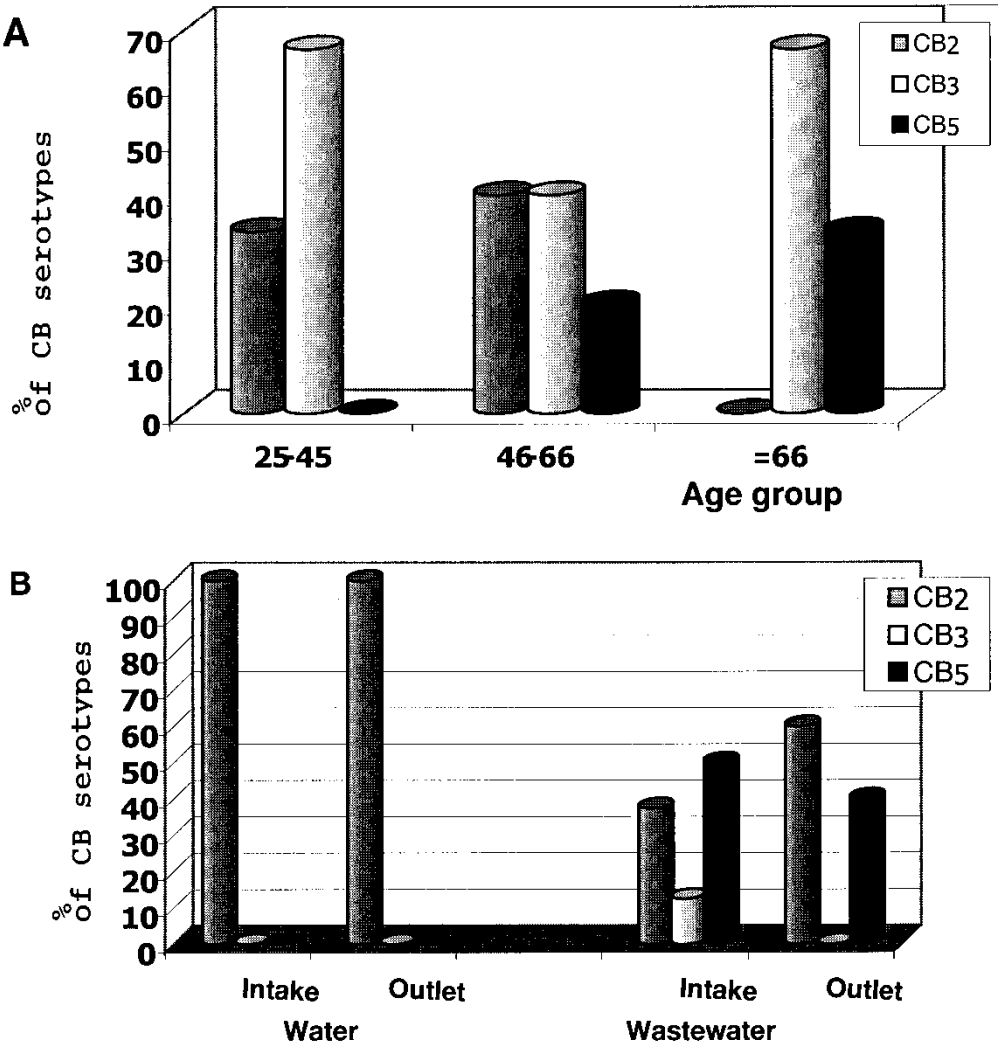

Fig. 1. Percentage of CB serotypes in (A) serum of acute myocarditis patients and (B) water and wastewater treatment plants at intakes and outlets.

viral myocardial infarction (MI) was assessed by serologic tests for anti-coxsackievirus specific antibodies (Roivainen *et al.* 1998) concluded that there was an age development relative risk of enterovirus MI correlated with high levels of antibodies that was maximum in middle-aged men (41 – 50 years old).

Similar observation of an increase of antibodies to coxsackieviruses group B in cases of MI were reported (Woods *et al.* 1975; Nicholls and Thomas, 1977; Nikoskelainen *et al.* 1983)

The present study covers both the source of virus transmission (water and wastewater) and health impact. Since myocarditis is an enigmatic condition to diagnose (Martin *et al.* 1994), integration of cell culture RT – PCR was used to increase virus titer to enhance detection. The difference in both direct RT – PCR to the serum samples and to the 1st passage cell culture

lysates was 34%. Detection of antibodies for enteroviruses is not significant because of the high antibody titer in our population for enteroviruses (Zeinab and Ali 1996). The most important observation in the results of the drinking water and wastewater treatment plants studied was the higher incidence of enteroviruses in the final treated water. This may be attributed to elution of accumulated viruses in the sand filter in addition to insufficient disinfection steps performed by chlorination or inadequate treatment processes.

Conclusion

Coxsackieviruses are the main etiologic agent of myocarditis. Water and wastewater are vehicles for these viruses. To diagnose the viral causative agent of myocarditis, it is recommended to integrate cell culture inoculation with RT – PCR. Water and wastewater treatment technologies could not eliminate the viral load of water completely. Accordingly, disinfection steps must be reviewed.

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