ENTERIC BACTERIA IN AQUATIC TURTLES

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ABSTRACT

Enteric bacteria were isolated from turtles of two lakes, one unpolluted and one contaminated with human wastes. Significant differences were found between the two samples of turtles with respect to total enterics isolated and distribution of enteric types. The "normal" enteric flora was found to be composed primarily of intermediate coliforms with representatives of other enteric groups also present.

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INTRODUCTION

Of the large number of reports dealing with the intestinal bacteria in reptiles, most have been concerned with the detection of members of the Enterobacteriaceae, in particular, Salmonella, Arizona, and related species. Interest in reptiles originated from the search for Salmonella which were not of human or domestic animal origin. Researchers often concentrated their studies in under-developed countries where sanitary conditions were lacking or where reptiles composed a portion of the people's diet (Maroja and Lowery, 1956; Mille, et al., 1959; Zwart, 1962). As information accumulated, the data indicated that reptiles had a strong capability to serve as vectors of Salmonella and Arizona (Fey, et al., 1957; Mille, et al., 1959; Edwards, et al., 1961; Zwart, 1962; Le Minor, et al., 1966; Bigland and Fox, 1966). Turtles, in particular, were found to harbor pathogenic forms for great lengths of time with no adverse effects to the host (Kiesewalter, et al., 1960; Boycott, 1962; Dimow, 1966; Ozek, et al., 1967; Rudat, et al., 1967).

With the incidence of salmonellosis having tripled in the United States between 1947 and 1964 (Kaufmann and Morrison, 1966), American epidemiologists began to search for new non-human reservoirs of Salmonella. British workers, however, were the first to associate a portion of the problem with pet turtles. Evidence indicated that the occurrence of enteric pathogens in turtles constituted a health hazard (Newman, 1966; Mann and Bjotvedt, 1967; Mann and Eaton, 1967).

As a result of the intensified studies of pathogenic bacteria in turtles, the epidemiologic role of these animals has been well established (Kaufmann, 1966; and Kaufmann and Morrison, 1966). But, in the eagerness to establish the presence of pathogens and/or to isolate and identify new serotypes, bacteriologists have failed to elucidate the role or define the nature of the overall bacterial flora found in turtles. This study will attempt to define the typical enteric flora of aquatic turtles from polluted and non-polluted sources with the idea of establishing the effect of the external environment as reflected by the intestinal flora. From the comparison of the Enterobacteriaceae of these two groups, it is hoped that the typical enteric flora can be defined and that the vector activities of turtles can be elucidated.

MATERIALS AND METHODS

Aquatic turtles were live trapped from two locations selected with respect to the degree of water pollution as indicated by the most probable number (MPN) of coliform organisms per 100 ml of water. Coliforms include all the aerobic and facultative anaerobic, non-spore-forming, gram-negative rods which ferment lactose with gas production within 48 hours at 35^{o}C (Geldreich, 1966). Beaver Lake in Pocahontas State Park, Chesterfield County, Virginia, served as the non-polluted source with a coliform count of 550. Lake Pasbehegh, James City County, Virginia, with a coliform count of 17,000-19,000, served as the polluted source. This lake has periodic influxes of human wastes as a consequence of malfunctioning septic fields in the surrounding housing development.

Both bottom dwelling and surface dwelling turtles were trapped to insure sampling of possible vertical stratifications of bacteria within the lakes. Bottom dwelling species collected were Sternothaerus odoratus, Kinosternum subrubrum and Chelydra serpentina. Surface dwelling species collected were Chrysemys picta and Pseudemys rubriventris.

A cloacal sample was obtained by aseptically injecting sterile water into the animal, extracting the wash and innoculating a nutrient broth tube. The innoculum was incubated overnight, diluted and streaked on nutrient agar and Salmonella-Shigella agar (SS agar, Difco).

Of the twenty-five colonies picked for examination, eight came from the SS agar and the remainder from the nutrient agar. The cultures were then screened to eliminate the non-enteric bacteria according to the morphological and biochemical definition of Edwards and Ewing (1967).

Gram staining and nitrate reduction tests followed procedures outlined in Benson (1967), and the oxidase test was completed according to directions for the cytochrome oxidase test in Collins (1967) with the substitution of N,N-dimethyl-p-phenylenediamine dihydrochloride for p-amino methyl aniline oxalate. After eliminating the non-enterics, cultures were IMViC classified (indole, methyl red, Vogues-Proskauer and citrate tests). The indole, methyl red and Vogues-Proskauer tests were performed as directed in Benson (1967) with the use of xylene and Ehrlich's reagent (instead of Kovac's) as suggested in Collins (1967). Citrate utilization was determined using Simmon's citrate agar (BBL).

Following IMViC classification, lactose fermentation was determined by a combination of two methods. Cultures were streaked on Levine's eosin methylene blue (EMB) agar (BBL) plus lactose and incubated 24 to 48 hours. Dark centered colonies indicated the fermentation of lactose, translucent colonies indicated no fermentation. To test for the presence of the enzymes responsible for lactose fermentation, the o-nitrophenyl-β-D-galactopyranoside (ONPG) test for β-galactosidase was employed as described by La Page and Jayaraman (1964). Heavy innocula were incubated in a medium containing 0.1%

ONPG (Sigma). If β-galactosidase was present, the colorless ONPG was hydrolyzed producing a readily discernible yellow color within several hours. This rapid reaction is opposed to the slow fermentation of lactose which often occurs in normal lactose media with many enteric bacteria which lack lactose permease but have β-galactosidase. Cultures which gave negative tests with EMB with lactose but gave a positive ONPG test were considered to be slow or late lactose fermenters.

The test for urease activity employed Christensen's medium without agar (Collins, 1967). After rapid urea hydrolysis had been noted in control cultures, the urease negative tubes were further incubated from four to eight days to separate the urease negative cultures from the late urease producers.

Hydrogen sulfide and motility tests followed the method of Gershman, et al., (1959). The test for phenylalanine deaminase was performed according to the procedure of Golnin and Glen (1962), and the lysine decarboxylase test followed the procedure of Kott (1962). Sensitivity to potassium cyanide was detected according to Møller as described in Collins (1967).

Carbohydrate fermentation (other than lactose) was determined using a peptone water medium (1.0% peptone and 0.5% NaCl) with a phenol red indicator and fermentable substrates incorporated from 0.5% to 1.0%. Durham tubes were used for the detection of gas from glucose and only with other carbohydrates as necessary.

The gelatin liquefaction test employed the innoculation of colonies into nutrient gelatin and incubation at 22°C for two days with observations for two weeks to detect slow protease activity.

Serological testing was used as confirmation for *Salmonella*, *Shigella* and *Escherichia*. These tests employed polyvalent typing antisera (BBL) by the simple slide agglutination technique.

Identification of Enterobacteriaceae (Edwards and Ewing, 1967) was used as the primary reference for the biochemical characteristics of the enteric groups and was supplemented by *The Bacteriology of the Enterobacteriaceae* (Kaufmann, 1969).

Statistical analysis employed a non-parametric test because of the assymetrical distribution of the data. The Kruskal-Wallis H Test (Woolf, 1968) was used to compare total enterics isolated from the two lakes and to compare total enterics isolated from top versus bottom dwelling species within each lake.

RESULTS

The number of enteric cultures isolated from the turtles from Beaver Lake and Lake Pasbehegh are given in Tables 1 and 2, respectively. These data indicate that the intestinal flora of animals from the two sources differ significantly with respect to members of the Enterobacteriaceae. In Lake Pasbehegh, alteration of the aquatic environment by human fecal contamination resulted in changes in microbial populations which were reflected in the number of enteric bacteria isolated from turtles of the two lakes. Animals trapped from Lake Pasbehegh had consistently lower levels of enteric bacteria per turtle than those from the unpolluted Beaver Lake. The Kruskal-Wallis H Test demonstrated the two samples to be significantly different at the 95% level when total enterics isolated were compared. Of 400 bacterial colonies isolated from Beaver Lake, 203 were enteric ($\bar{X}$=12.7/turtle); of 475 colonies isolated from Lake Pasbehegh, 125 were enteric ($\bar{X}$=6.5/turtle).

Table 3 shows the distribution of enteric bacteria isolated from each species of turtle collected. The distribution of enteric types showed a marked difference between the two lakes. In Beaver Lake, intermediate coliforms comprised the majority of the flora (70.4%) with the next largest group being *Proteus* (8.8%). Of the intermediates, IMViC types ++-+ (23.1%) and +--+ (24.1%) were predominant. The remaining enterics were distributed as follows: *Klebsiella* (4.9%), *Enterobacter* (5.4%), *Escherichia* (4.4%),

TABLE 1. Number of enteric cultures isolated from the turtles of Beaver Lake

Species	Total colonies isolated	Number enteric
Sternothaerus odoratus	25	2
Sternothaerus odoratus	25	5
Sternothaerus odoratus	25	22
Sternothaerus odoratus	25	9
Sternothaerus odoratus	25	24
Sternothaerus odoratus	25	21
Sternothaerus odoratus	25	19
Sternothaerus odoratus	25	24
Kinosternum subrubrum	25	3
Chrysemys picta	25	4
Chrysemys picta	20	15
Chrysemys picta	25	8
Chrysemys picta	25	11
Pseudemys rubriventris	30	21
Pseudemys rubriventris	25	10
Pseudemys rubriventris	25	8
Total	400	203
		$\bar{X}$=12.7

TABLE 2. Number of enteric cultures isolated from the turtles of Lake Pasbehegh

Species	Total colonies isolated	Number enteric
Kinosternum subrubrum	25	15
Kinosternum subrubrum	25	11
Kinosternum subrubrum	25	0
Kinosternum subrubrum	25	2
Kinosternum subrubrum	25	0
Kinosternum subrubrum	25	11
Kinosternum subrubrum	25	2
Kinosternum subrubrum	25	14
Kinosternum subrubrum	25	3
Kinosternum subrubrum	25	0
Kinosternum subrubrum	25	2
Kinosternum subrubrum	25	11
Chelydra serpentina	25	5
Chrysemys picta	25	1
Chrysemys picta	25	0
Chrysemys picta	25	12
Chrysemys picta	25	17
Chrysemys picta	25	11
Pseudemys rubriventris	25	8
Total	475	125
		$\bar{X}$=6.5

TABLE 3. Number of cultures isolated per enteric genus from each species of turtle collected from Beaver Lake and Lake Pasbehegh[a]

	Klebsiella	Enterobacter	Escherichia	Proteus	Providence	Citrobacter	Intermediates (all types)	Intermediate (++-+)	Intermediate (+--+)	Total
Beaver Lake:										
Sternothaerus (8)	5	10	9	7	5	2	85			123
Kinosternum (1)	0	0	0	0	0	0	3			3
Chrysemys (4)	0	1	0	5	3	2	27			38
Pseudemys (3)	5	0	0	6	0	0	28			39
Total	10	11	9	18	8	4	143	(47)	(49)	203
Percentage	4.9	5.4	4.4	8.8	3.9	1.9	70.4	(23.1)	(24.1)	
Lake Pasbehegh:										
Kinosternum (12)	20	19	9	3	1	0	19			71
Chelydra (1)	5	0	0	0	0	0	0			5
Chrysemys (5)	8	5	4	4	0	0	20			41
Pseudemys (1)	1	2	0	0	0	0	5			8
Total	34	27	13	7	1	0	45	(12)	(3)	125
Percentage	27.1	20.8	10.4	5.6	0.8	0.0	35.2	(9.6)	(2.4)	

[a]The sample size is given following each turtle genus.

Providence (3.9%), Citrobacter (1.9%). In the polluted lake, there was a greater diversity with Klebsiella (27.1%), Enterobacter (20.8%), Escherichia (10.4%) and the intermediates (35.2%) composing 93.5% of all enterics. Of the intermediates types ++-+ and +--+ decreased to 9.6% and 2.4% of the total isolates. The balance of the enterics were Proteus (5.6%) and Providence (0.8%).

The Kruskal-Wallis H Test was used to test for differences in the number of enterics isolated from top versus bottom dwelling turtles. Comparisons of Chrysemys and Pseudemys against Sternothaerus and Kinosternum from Beaver Lake resulted in no significant difference in total colonies of Enterobacteriaceae between the two groups. Similarly, in Lake Pasbehegh, Chrysemys and Pseudemys versus Kinosternum and Chelydra resulted in no significant difference. This test constituted a comparison of the two groups as ecological types rather than as a comparison between host species.

No species specific relationships between hosts and bacteria could be detected. (See Appendix for distribution of bacteria in individual animals.)

A checklist of the Enterobacteriaceae from all turtles sampled is given in Table 4. No Salmonella, Arizona or Shigella were isolated from the turtles sampled. Although SS agar was employed, this was only after the cloacal samples had been incubated overnight in nutrient broth. No enrichment media were used since the procedures were designed to isolate pathogens only if they constituted a large percentage of the intestinal flora. The vector potential of turtles as carriers of pathogenic Enterobacteriaceae has been well established by previous work.

TABLE 4. Checklist of Enterobacteriaceae among all turtles sampled

	Shigella	*Escherichia*	*Salmonella*	*Arizona*	*Citrobacter*	*Klebsiella*	*Enterobacter*	*Hafnia*	*Serratia*	*Proteus*	*Providence*
Sternothaerus	-	+	-	-	+	+	+	-	-	+	+
Kinosternum	-	+	-	-	-	+	+	-	-	+	+
Chelydra	-	-	-	-	-	+	+	-	-	-	-
Chrysemys	-	+	-	-	+	+	+	-	-	+	+
Pseudemys	-	-	-	-	-	+	+	-	-	+	-

DISCUSSION

These data suggest that the typical enteric flora of aquatic turtles from unpolluted environments would consist predominantly of intermediate coliform types with representatives of the Proteus-Providence, Klebsiella-Enterobacter and Escherichia groups. The presence of significant fecal contamination from warm blooded animals would alter the relative frequencies of the above mentioned groups and introduce the potential of contamination by pathogenic members of the Enterobacteriaceae. Very few reports exist in the literature concerning enteric bacteria in turtles, other than pathogens. Capponi, et al., (1956), reported Proteus, Escherichia, Citrobacter (Bethesda-Ballerup Group), Providence, Serratia, Salmonella and Arizona from geckoes and tortoises. More recently, Jackson, et al., (1969), reported the preliminary results of a survey of enteric bacteria in captive and wild turtles from the southern United States. In addition to several serotypes of Salmonella, Citrobacter (Bethesda-Ballerup Group) was isolated from Kinosternum, Pseudemys and Chrysemys picta. Escherichia and Enterobacter were also reported but with no reference to the host species.

Turtles from water with the higher coliform count had a lower count of enteric bacteria than turtles from the lake with the lower coliform count. Thus, contrary to expectations, there may not be a positive correlation between the enteric intestinal flora and the environment. The difference observed in Lake Pasbehegh animals can best be clarified by considering the effect of human fecal contamination in the aquatic environment. With an influx of human wastes, the spectrum of bacteria present in the water is likely to be altered. An increase in the number of coliforms occurs coupled with increases in the numbers of fecal streptococci and certain anaerobic spore-forming baccilli. These last two groups, common bacterial components of human feces, are not detected by coliform counts, and, consequently, the percentages of these organisms in the water is not known unless specifically tested. Geldreich (1966) reports that fecal streptococci may be found in numbers equal to coliforms in density measurements of sewage.

From a study of salmonellosis in turtles, Kaufmann and Morrison (1966) have proposed that "the level of contamination of pond water does not reflect the level of contamination of the adult turtle." These workers made this statement based on results of an examination of two turtle breeding ponds, one with high and one with low salmonellae contamination. Turtles from both ponds were negative for salmonellae in the intestines. However, from the high level pond Salmonella was isolated from the ovaries of one animal and the gall bladder of another (ten animals sampled). On the other hand, from the low level pond, eight of ten turtles were infected with salmonellae, seven positive for the ovaries and one positive for the gall bladder. Consequently, in light of this data, results from the comparison of Beaver Lake and Lake Pasbehegh appear to follow Kaufmann and Morrison's proposal. The more highly contaminated lake (Lake Pasbehegh) had fewer enterics isolated per turtle than the unpolluted lake.

The increase in numbers of fecal bacteria relative to coliforms in the polluted lake provides a possible explanation for the lower number of enterics isolated per turtle in Lake Pasbehegh. Since there is no source of human contamination in Beaver Lake, it can be assumed that streptococci and anaerobic spore-forming baccilli of fecal origin would be present in low concentrations or not at all. Thus, the changes in bacterial spectrum from one lake to the other may be reflected in the number of enterics isolated per turtle, in this case, a reduction. The lower count in Lake Pasbehegh animals might be a result of changes in competition of bacterial types for niches in the intestinal ecosystem, i.e., there is a change of interaction between the internal and external environments.

Analysis of biochemical types of enteric bacteria gives a good correlation between the enteric types expected for the two types of aquatic environments. In animals from Lake Pasbehegh, greater than 50% of the isolates were _Escherichia_ (10.4%), _Enterobacter_ (20.8%) and _Klebsiella_ (27.1%), bacteria commonly associated with human fecal contamination. Geldreich (1966) has shown that IMViC types ++-- and --++ are the predominant coliform types found in polluted water. These types correspond to _Escherichia_ (++--), _Enterobacter_ (--++) and _Klebsiella_ (--++). In Beaver Lake, however, this group comprised only 14.7% of all enterics isolated; the predominant forms were the intermediate coliforms (70.4%) with the fecal coliforms forming only a small percentage of the total enterics isolated: Escherichia (4.4%), Enterobacter (5.4%) and Klebsiella (4.9%).

In Beaver Lake, the predominance of the intermediates was due primarily to two types: ++-+ (23.1%) and +--+ (24.1%). Animals from Lake Pasbehegh, however, had markedly fewer intermediates (35.2%) with a sharp drop of types ++-+ and +--+ to 9.6% and 2.4%, respectively. Geldreich (1966) has demonstrated a correlation between the number of intermediates and the nature of the aquatic environment. This author reported that 51.6% of the coliforms in unpolluted water were of the intermediate type -+-+ with the fecal types found only in low numbers. Coliforms isolated from unpolluted soil were also predominantly -+-+ and the author suggests a correlation between soil and water bacteria was due to soil washing as a contributing factor to the flora of the lake. Data reported here supports Geldreich's (1966) study in respect to both fecal and intermediate coliform types. It can be assumed that the predominance of intermediate types isolated from Beaver Lake were initially soil in origin. Supporting this was the next highest group isolated which was Proteus, a common soil bacteria. The correlation is further enhanced by the drop in intermediate coliforms from 70.4% in Beaver Lake to 35.2% in Lake Pasbehegh and a reduction in Proteus from 8.8% to 5.6%.

Inferences with respect to bacterial genus specificity to host species are difficult to make because of small sample numbers and a limited number of isolates for many samples. As indicated by Tables 3 and 4, Enterobacter, Klebsiella, Escherichia and Proteus are fairly well represented in most turtle species sampled. Providence, although present in isolates from both lakes, is represented by only one isolate in Lake Pasbehegh, and Citrobacter was found only in Beaver Lake. The lack of numerically significant data, however, prevents any definite conclusions from being made. (See Appendix for distribution of types within individual turtles.) Kaufmann and Morrison (1966) have noted, however, that Salmonella rubislaw, a pathogen, has demonstrated an affinity for reptiles as hosts. Consequently, the existence of species specific interrelation is feasible.

The "normal" enteric flora can be defined as being composed primarily of intermediates reflecting the bacterial nature of the water and surrounding soil. An influx of pollution, however, may alter this flora. Results from this study show a positive correlation of Enterobacteriaceae to their hosts in an aquatic environment. This correlation, however, is not necessarily with absolute numbers of enterics in the water with the number of enterics isolated from the animal, rather, it is with the biochemical types.

APPENDIX

APPENDIX A

Distribution of Enteric Types in Individual
Turtles Sampled: Beaver Lake

Turtle Number	Species	Total colonies isolated	Number enteric	Genera		Number
3	Pseudemys rubriventris	30	21	Klebsiella		5
				Intermediates:	(-+--)	1
					(++-+)	8
					(-++-)	4
					(+--+)	2
					(-+++)	1
4	Sternothaerus odoratus	25	2	Klebsiella		1
				Enterobacter		1
5	Sternothaerus odoratus	25	5	Enterobacter		2
				Intermediates:	(++-+)	1
					(+--+)	2
6	Sternothaerus odoratus	25	20	Klebsiella		1
				Intermediates:	(+-++)	2
					(++-+)	3
					(+-+-)	2
					(---+)	2
					(+--+)	9
					(-+--)	1
7	Sternothaerus odoratus	25	9	Klebsiella		1
				Intermediates:	(+--+)	5
					(---+)	1
					(+---)	1
					(+++-)	1
8	Chrysemys picta	25	4	Intermediates:	(+-++)	1
					(---+)	3

9	*Sternothaerus odoratus*	25	23	*Proteus*		4
				Providence		2
				Klebsiella		2
				Escherichia		2
				Intermediates:	(++-+)	5
					(+--+)	6
					(---+)	1
					(+-++)	1
10	*Sternothaerus odoratus*	25	21	*Escherichia*		5
				Enterobacter		3
				Providence		1
				Intermediates:	(++-+)	3
					(+--+)	6
					(---+)	1
					(-+--)	1
					(+-++)	1
11	*Sternothaerus odoratus*	25	24	*Escherichia*		2
				Proteus		2
				Providence		2
				Intermediates:	(+-++)	10
					(++-+)	5
					(---+)	3
12	*Sternothaerus odoratus*	25	19	*Enterobacter*		4
				Proteus		1
				Citrobacter		2
				Intermediates:	(++-+)	7
					(+--+)	3
					(++++)	1
					(+-++)	1
18	*Chrysemys picta*	20	15	Intermediates:	(+--+)	7
					(++-+)	8
27	*Pseudemys rubriventris*	25	8	Intermediates:	(+--+)	3
					(++-+)	4
					(+---)	1
28	*Kinosternum subrubrum*	25	3	Intermediates:	(+---)	1
					(++-+)	1
					(----)	1
30	*Chrysemys picta*	25	8	*Proteus*		2
				Citrobacter		2
				Enterobacter		1
				Intermediates:	(+--+)	1
					(++-+)	1
					(----)	1

34	Chrysemys picta	25	11	Proteus	3
				Providence	3
				Intermediates: (+-++)	1
				(++-+)	1
				(+--+)	2
				(---+)	1
37	Pseudemys rubriventris	25	10	Proteus	6
				Intermediates: (+--+)	1
				(-+-+)	1
				(---+)	1
				(+-++)	1

APPENDIX B

Distribution of Enteric Types in Individual
Turtles Sampled: Lake Pasbehegh

Turtle Number	Species	Total colonies isolated	Number enteric	Genera		Number
1	*Kinosternum subrubrum*	25	15	*Enterobacter*		11
				Klebsiella		1
				Intermediates:	(----)	2
					(+++-)	1
2	*Kinosternum subrubrum*	25	11	*Enterobacter*		3
				Klebsiella		5
				Escherichia		1
				Intermediates:	(----)	1
					(+-++)	1
4	*Chelydra serpentina*	25	5	*Klebsiella*		5
5	*Kinosternum subrubrum*	25	2	Intermediates:	(+-++)	1
					(+---)	1
7	*Chrysemys picta*	25	1	*Enterobacter*		1
8	*Kinosternum subrubrum*	25	11	Providence		1
				Escherichia		3
				Intermediates:	(---+)	3
					(----)	2
					(+--+)	2
9	*Kinosternum subrubrum*	25	2	*Enterobacter*		1
				Escherichia		1
10	*Kinosternum subrubrum*	25	14	*Klebsiella*		13
				Proteus		1
11	*Kinosternum subrubrum*	25	3	*Escherichia*		3

13	*Kinosternum subrubrum*	25	2	*Escherichia*	1
				Intermediates: (+---)	1
15	*Pseudemys rubriventris*	25	8	*Klebsiella*	1
				Enterobacter	2
				Intermediates: (---+)	3
				(++-+)	1
				(+--+)	1
16	*Chrysemys picta*	25	12	*Klebsiella*	8
				Enterobacter	1
				Intermediates: (---+)	3
17	*Kinosternum subrubrum*	25	11	*Proteus*	2
				Klebsiella	1
				Enterobacter	4
				Intermediates: (++-+)	2
				(---+)	1
				(++++)	1
18	*Chrysemys picta*	25	17	*Proteus*	2
				Escherichia	4
				Intermediates: (++-+)	9
				(+-++)	1
				(---+)	1
19	*Chrysemys picta*	25	11	*Proteus*	2
				Enterobacter	3
				Intermediates: (---+)	6

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