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CORRELATION OF NATURALLY OCCURRING CONCENTRATIONS OF FREE-AMMONIA IN THE CAECUM OF RATS WITH RESISTANCE TO EXPERIMENTAL *ENTAMOEBA HISTOLYTICA* INFECTION

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YOUNG rats are generally used as experimental hosts for *Entamoeba histolytica* infection in virulence and chemotherapeutic studies. Infection is induced according to Jones[1] by directly inoculating the amoebae into the caecum at the time of laparotomy. The development of caecal amoebiasis of each infected rat is graded by criteria described in literature[1–3]. The average degree of infection (ADI) provides the virulence status of the parasite. A strain of *E. histolytica* is considered virulent if it can cause caecal mucosal ulceration.

It has often been recorded that despite similar test procedures, differential development of *E. histolytica* infection occurs and in some rats, the amoebae fail altogether to colonize. The factors that have received serious attention in the experimental production of *E. histolytica* infection include the quality of amoebic inocula, bacterial factors and dietary status of the host[4].

In earlier studies[5,6], the caecum of rats with faecal pH 5.5–6.5 was found to be susceptible to *E. histolytica* infection and not those with pH 7.5. In this communication the above results are further substantiated and the death of these amoebae in the caecum of rats bearing alkaline contents correlated with the naturally occurring concentration of free ammonia.

Two strains of *E. histolytica*, B-1 and NIH-200 were used in this study. The former was freshly isolated from an acute case of human amoebiasis and maintained under xenic condition in modified Boeck and Drabohlav medium[7]. The latter was maintained axenically in modified TPS-1 medium[8].

Weanling albino rats (21-day old) (Druckrey), weighing 18–20 g, were obtained from the stock colony maintained at the Animal House of the Institute. Only those rats with faecal pH 5.5–6.5 or 7.5 were used for inoculation and the rest were discarded. The procedures used in determining the faecal and caecal pH and also in the production and evaluation of caecal amoebiasis of rats have been reported earlier[5].

The results (table 1) show cent per cent development of caecal amoebiasis in animals possessing faecal pH 5.5–6.5. In contrast only 2 out of 20 with faecal pH 7.5 developed *E. histolytica* infection. Significantly, the caecal contents of rats positive for *E. histolytica* infection possessed pH 7.5. The whitish adhesions on the caecal wall containing a very large number of trophozoites of *E. histolytica* possessed pH 6.5.

An attempt was made to identify the factors which could be responsible for the failure of *E. histolytica* to infect rats possessing caecal content of alkaline pH. Studies were carried out to determine the *in vitro* amoebicidal action of caecal contents of rats. The contents from each rat failing to develop the amoebic infection or normal ones were collected, suspended in 2.5 ml distilled water and their pH recorded. These were then added promptly to young actively multiplying culture of *E. histolytica* (B-1) growing in 2.5 ml monophasic culture medium (Inactivated bovine serum was diluted 1:7 with M/40 phosphate buffer containing 0.85% NaCl and particulate rice starch). Tests were carried out at pH 6.5 and also at pH 7.5 adjusted with N/10 NaOH.

Table 1 *Caecal amoebiasis of rats in relation to faecal pH.*

Range of faecal Ph	No. of rats inoculated	No. of rats with ulcers		Av. caecal score
	infected	3-grade	4-grade	
5.5–6.5	46/46	18	21	6.3
7.5–7.8	20/2	Nil	Nil	0

Microscopic examination was made after 24 hr to determine the viability of the amoebae. Some tubes in these tests were also treated with acriflavin and gentian violet (each in the conc. of 1:10,000). The effect of aqueous extracts of the caecal contents on trophozoites of *E. histolytica* was also determined. The extracts were obtained by centrifuging 2.5 ml of a chilled aqueous suspension of caecal contents at 3000 r.p.m. for 1 hr. The supernatant thus obtained was used after addition of penicillin (1000 μg/ml) and streptomycin (1000 μg/ml).

The results in all these tests were much similar. The amoebae remained well protected in media at pH 6.5 when caecal contents or extracts were added. The amoebae died in tests carried out in media at pH 7.5. The addition of antiseptic dyes promoted vigorous growth of amoebae where the cultures remained viable. These results indicate the possible occurrence of factor(s) in the rat caecum possessing pH-dependent antiamoebic activity.

An analysis of the caecal contents of rats failing to develop *E. histolytica* infection or of normal ones has revealed the consistent occurrence of free-ammonia only under alkaline conditions. Ammonia vapours from the caecal contents were absorbed in N/50 HCl. These solutions were reacted with Nessler's reagent and ammonia estimations made colorimetrically.

The caecum of rats containing copious amounts of solid material was found to contain free ammonia in concentration of 18–25 μg/g. Significantly, the mucoidal contents of grossly ulcerated caecum did not contain detectable quantities of free ammonia. The status of caecal and faecal pH of rats in relation to ammonia contents and weight of the animals is presented in table 2. The weight of the animals was not found to correlate with the nature of pH condition.

A series of studies were carried out to determine the amoebicidal property of free ammonia. The volatile materials from the caecal contents of rats liberated under alkaline condition and containing free-ammonia was bubbled through cultures of axenic *E. histolytica* growing at pH 6.5 and 7.2 in specially fabricated culture vessels. Blanks and ammonia vapours from urea was included as control. The tubes were examined under an inverted microscope for viable amoebae at intervals of 24 hr for 3 days.

The amoebae in test cultures at pH 7.2 started detaching from the sides of the tube and could be seen in a free floating condition by 24 hr. A majority of dead amoebae in crumpled and shrunk conditions was found settling at the bottom of the tubes by 72 hr. Similar results were obtained when the source of ammonia was urea instead of caecal contents of rats. However, in control cultures, irrespective of pH and in tests conducted in media at pH 6.5, the amoebae remained unaffected.

The amoebicidal action of ammonia was also distinguished from the inhibitory action of adverse pH conditions by the following methods.

1. Modified TP-S-1 medium was prepared at pH 5, 5.5, 6, 6.5, 7, 7.5, 8 and 8.5 with the aid of N/10 NaOH. NaCl was not added so as to prevent precipitation of media constituents under alkaline conditions The ability of *E. histolytica* to grow through three subcul-

Table 2 *The pH and ammonia concentrations of the faecal pellet and caecal contents of rats in relation of weight of the animal.*

Rats	Weight (g)	Faecal pH	Pellet ammonia (μ/g)	Caecal pH	Contents ammonia (μ/g)
1	28	7.0	Nil	6.89	Nil
2	20	6.06	Nil	6.89	Nil
3	30	6.90	Nil	7.70	19
4	26	7.23	Nil	6.92	Nil
5	39	6.43	Nil	6.73	Nil
6	25	7.20	Nil	6.85	Nil
7	18	7.61	18	7.34	Nil
8	22	7.73	23	7.27	Nil
9	27	7.54	25	7.72	23
10	23	7.21	Nil	6.90	Nil
11	39	7.91	25	7.83	25
12	30	7.81	23	7.61	21
13	27	7.86	17	7.59	17
14	30	7.17	Nil	7.45	Nil
15	27	6.01	Nil	5.96	Nil

tures were recorded. The cultures were seeded always with 10,000 amoebae per ml of the medium and subcultures made at 72 hr.

Absence of NaCl from the modified TP-S-1 medium apparently did not affect the proliferation of *E. histolytica*. The amoebae grew well in media bearing pH upto 7. At pH 7.5, though the amoebae remained viable, they failed to proliferate and could not be carried till the third subculture. In media bearing pH 8 and 8.5, the amoebae failed to adhere to the sides of the test tube and died out by 72 hr. These media were found to contain free ammonia at the concentration of 0.4 μg/ml.

2. In this test, modified TP-S-1 medium of pH 7.5 was prepared with the addition of N/10 NaOH or NH_4OH and its suitability in supporting the growth of *E. histolytica* determined. The amoebae were found adhering to the sides of the test tube in media containing NaOH but not in those containing NH_4OH. The latter tubes contained 10–25 μg/ml of free-ammonia.

3. The toxicity of free-ammonia on trophozoites of *E. histolytica* was also tested by studying the *in vitro* action of urea on these amoebae in media at pH 6.5 and 7.2. Tests were conducted by the method of Das and Krishna Prasad[9]. The results also show pH-dependent toxicity of urea. The amoebae failed to survive at pH 7.2 in media containing urea, in concentrations of 15.6 μg/ml. At pH 6.5, however, no toxic effects were noticed even upto 1000 μg/ml of urea.

In the studies described above, the phenomenon of natural resistance of the rat caecum to experimental *E. histolytica* infection has been correlated with the toxic action of free-ammonia under alkaline conditions.

The inhibitory action of adverse pH conditions on the *in vitro* proliferation of *E. histolytica* is well documented[10–14]. Our studies point to the relationship between free ammonia in the culture media possessing alkaline pH, and their inability to support growth of these amoebae.

Albach and Shaffer[15] have reported that the ammonia produced by *Bacteriodes* sp in CLG medium did not affect the growth of *E. histolytica* because the pH of the medium remained stable at 5.8 both prior to and during the proliferation of these amoebae. Warren and Schenker[16] have suggested that change in pH alters the toxic effects of ammonia. This can be explained by the fact that ammonia cannot occur in a free state under acidic condition. Our studies with urea also indicate the inefficacy of ammonium compounds (urea) in killing the trophozoites of *E. histolytica* in media bearing acidic pH.

It can be pointed out here that the pH of pathological materials positive for *E. histolytica* infection have a tendency towards acidic range[17, 18]. Also the disappearance of the dysentery amoebae that was correlated with the changed chemical composition of the stools, especially in regard to increased concentration of ammonia as reported by Lösch[19] finds adequate support from our studies.

In conclusion it should be emphasized that any approach dealing with virulence or chemotherapuetic studies must also consider the pH condition of the contents of the large intestine. Since the pH of the caecal contents of rats varies, it obviously will result in dissimilar development of *E. histolytica* infection and also (possibly) in response to therapeutic agents.

The authors are thankful to Dr Nitya Nand, Director, Dr N. M. Khanna, Dy. Director and Incharge, Pharmaceutics Division and Dr S. K. Gupta (Retd) Ex-incharge, Microbiology Division for their interest and encouragement in carrying out this work.

23 August 1983

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ISOLATION OF *SALMONELLA LIMETE* AFTER TWO DECADES IN INDIA

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DURING an eco-epidemiological survey of *Salmonella* in and around Manipal a west coastal region of Karnataka, South India, a large number of *Salmonella* isolations made from 125 healthy frogs (*Rana pipiens*) revealed that they are of known pathogenicity to human beings. Of 73 *Salmonella* isolates belonging to 9 different serotypes[1], 7 strains of *Salmonella limete* (1, 4, 12, 27:b:1, 5) were recovered between August and December 1982. All the strains were recovered from the intestines of healthy frogs. The frogs were collected from wells, rivers, ponds and streams present within a radius of 15 km from Manipal. Rappaport's medium and modified MacConkey's agar were mainly used for isolation. The strains were identified by standard procedures[2,3] and serotyped at Central Research Institute, Kasauli and Adelaide, Australia. The antibiogram revealed their sensitivity to most of the commonly used drugs like, ampicillin, chloramphenicol, gentamycin, kanamycin, streptomycin, cotrimoxazole and polymyxin.

So far, a single strain of *S. limete* has been isolated only once in India at Hissar from a cattle[4]. Our isolation at Manipal becomes the first report of recovery of *S. limete* from South India, being isolated exactly after 2 decades of its first isolation at Hissar (North India) in November, 1962 (Saxena, S. N. Personal communication).

Frogs are the free living amphibians which survive in close association with human beings. Being present in livestock premises and human dwellings, they act as chief reservoirs of pathogenic Salmonellae in nature. They can contaminate the water of rivers, ponds and even wells of rural areas and thus pose serious health hazards.

26 April 1983; Revised 16 January 1984

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STOMATAL PECULIARITIES IN *CATHARANTHUS ROSEUS* (LINN.) G. DON (APOCYNACEAE)

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WHILE undertaking a detailed study of morphology and ontogeny of stomata in some members of this family, the author came across unique types of foliar stomata in *Catharanthus roseus* (Linn.) G. Don which are hitherto unreported in this plant as well as in the family Apocynaceae. Metcalfe and Chalk[1] reported only two types of stomata: ranunculaceous and rubiaceous in this family. Kapoor *et al*[2] recorded shrivelled stomata (abnormal type) in *Thevetia peruviana* (Pers.) Schum. and Patel *et al*[3] recorded six kinds of abnormal stomata in the leaves of *Aganosma dichotoma* (Roth.) K. Schum.

Leaves were collected from plants growing in different localities of Delhi, and were boiled in 2% HNO_3 for 7–10 min. The epidermal peels thus obtained were