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Etiology of the 1965 epidemic of febrile illness in Nagpur City, Maharashtra State, India

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An investigation of an extensive outbreak of febrile illness during the months of April, May, and June 1965, in the city of Nagpur, Maharashtra State, showed that the main etiological agent was chikungunya virus. Dengue type 4 and Chandipura viruses were also active during this period. In all, 26 strains of virus were isolated from 60 acute phase human sera, and of these strains, 23 were identified as chikungunya virus, 2 as Chandipura, and 1 as dengue type 4. Five strains of chikungunya virus and 9 strains of dengue type 4 virus were isolated from 34 pools of Aedes aegypti *collected from the affected areas. Results of complement fixation tests with acute–convalescent paired serum samples and single convalescent sera confirmed that chikungunya virus was the main etiological agent. The significance of these findings is discussed.*

In 1965, the city of Nagpur in Maharashtra State experienced an extensive epidemic of febrile illness characterized mainly by severe joint and body pains. The epidemic appeared to commence in the first week of April, reached its peak during the latter half of the month and the first three weeks of May, and then gradually declined. Towards the end of June only sporadic cases were detected. A very rough estimation showed the case incidence to be higher than 50% of the population in certain highly congested areas.

An investigation of the etiology of the epidemic was conducted by the Virus Research Centre (VRC) between 5 June and 23 June 1965 in co-operation with the health authorities of the Nagpur Municipal Corporation and the State Department of Public Health. This report presents the results of these investigations.

MATERIALS AND METHODS

Collection and transport of sera

Blood samples were collected by venepuncture and were stored at approximately 4°C until the serum was separated from the clot, 24–72 h after collection. At first, sera collected during the acute stage of the illness (1–8 days after the onset of illness) were stored at −50°C for a few days and were then transported on solid carbon dioxide to the VRC at Poona. Later this practice was discontinued and the acute sera were stored at 4°C for a few days and were transported on ice to the VRC in the same way as were the convalescent sera (collected from day 9 onwards) and the sera collected from the contacts of cases.

Collection and transport of mosquitos

Mosquitos were collected by hand from human dwellings in the affected areas. Live female *Aedes aegypti* were sorted and kept in Barraud cages (other mosquitos were discarded) and were transported twice weekly, by air, to the VRC, where the identification was confirmed and the mosquitos were divided into pools of 4–50.

Processing of specimens, inoculation into mice, and observations

Immediately on arrival at the VRC, each undiluted acute serum was inoculated in 0.02-ml doses intracerebrally into two litters (8 mice per litter) of 2- to 3-day-old Swiss albino mice. The mice were the progeny of a stock received from the Rockefeller Foundation Laboratories, USA, and maintained at the VRC.

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The method of preparation of mosquito pool suspensions was similar to that followed by Pavri & Singh (1965). The suspensions were inoculated intracerebrally into 2- to 3-day-old mice.

Mice were observed for 16–21 days for signs of illness. Passages were made from the brains of sick mice, whenever necessary.

Virus isolation in tissue culture

BS-C-1 cells, primary bonnet monkey (*Macaca radiata*) kidney cells (MKTC), and/or BHK-21 cells were used in attempts to isolate the virus from undiluted serum samples. The methods of preparation and maintenance of the cells, inoculation of the material, and observation of the tubes were those described by Bhatt & Rodrigues (1967).

Titration of chikungunya virus in human sera

In order to determine the concentration of chikungunya virus in sera during the acute phase of the illness, the virus present in 8 sera was titrated in infant mice by the intracerebral route: 6 of these sera were also titrated in BS-C-1 cells. The sera had been stored at −50°C for 6–17 days following the initial isolation attempt.

Cross-challenge tests in mice

These tests were performed according to the method described by Paul et al. (1965) in those cases where 4 or more mice survived from the mouse groups inoculated with any individual serum. The mice were challenged 15–22 days after inoculation with a dose of 1 200 LD_{50} or 1 600 LD_{50} of the TR 1751 strain of dengue type 2 virus.

Identification of strain No. 653705 by neutralization tests in tissue culture

The method employed was similar to that described by Paul, Banerjee & D'Lima (1965). The medium used was Eagle's basal medium with 3% fetal calf serum. The tubes were challenged with 1 000 $TCID_{50}$ of type 1 poliovirus. The results were read as described by Paul & Banerjee (1965).

Complement fixation tests

" Quick " complement fixation (QCF) tests for identification of the virus were carried out as described by Pavri & Sheikh (1966).

Serum samples were tested by the complement fixation (CF) test, according to the method described earlier (Pavri et al., 1962) with antigens of Japanese encephalitis (JE), dengue type 1 (DEN-1), dengue type 2 (DEN-2), Kyasanur forest disease (KFD), Sindbis (SIN), and chikungunya (CHIK) viruses, prepared according to the techniques described by Clarke & Casals (1958). A few sera were also tested with dengue type 3 (DEN-3) and dengue type 4 (DEN-4) antigens. A 4-fold or higher rise in titre of CF antibodies in the convalescent serum sample as compared with the titre in the acute sample was considered as evidence of infection with the respective virus(es). The figures in the tables pertaining to the results of CF tests represent the reciprocals of the highest dilutions of the respective sera that fixed approximately 2.5 units of complement.

DESCRIPTION OF ILLNESS [1]

The disease affected all age groups but the incidence appeared to have been highest in young adults and lowest in children. No appreciable difference in incidence was noticed between the sexes.

The illness usually had a sudden onset either with fever or with body and joint pains followed soon after by fever. Rarely a prodromal phase of malaise was present a few hours before the fever. Joint pains, often accompanied by swelling, were present in most of the cases, the joints commonly affected being the interphalangeal, knee, ankle, and wrist joints. The maximum temperatures ranged between 37.8°C and 41.1°C.

The fever lasted for a period of 1–7 days, the usual duration being 2–4 days. In some cases the fever was accompanied by chills. Myalgia, pain in the lumbar region, headache, marked debility, vertigo, anorexia, and vomiting were the other symptoms present.

In some cases a morbilliform rash distributed chiefly over the trunk and limbs appeared between the second day and the fifth day after onset, in some instances when the fever had begun to subside. The rash lasted from a few hours to 3 days.

A number of patients had a recurrence of fever after an apyrexial period that varied from 1 to 10 days, the usual interval being 3–7 days. The second attack was milder than the first.

Haemorrhagic manifestations were uncommon. Cases with bleeding gums or epistaxis were occasion-

[1] This picture of the illness is based mainly on the description given by physicians at Municipal dispensaries (including one of the authors—MRP), at the Employees State Insurance Scheme dispensaries, and at hospitals, as well as a few in private practice. The physicians had the experience of treating numerous cases. The symptomatology of the cases from whom specimens were obtained was in general agreement with this description (no physical examination was carried out on these latter cases).

ally seen. Only rarely were cases with haematemesis, vaginal bleeding, or bleeding per rectum reported.

There were few physical signs. Enlarged lymph nodes were seen in a number of cases chiefly in the cervical region. Inguinal and posterior auricular glands were also enlarged. The lymph nodes in some cases were tender. A common finding was the presence of stomatitis with, in some cases, vesicles present on the palate, fauces, and the posterior pharangeal wall. Gingivitis was also fairly commonly noticed. Enlargement of the spleen was sometimes detected.

Convalescence was usually prolonged and was characterized by marked weakness and by pain in the joints, which lasted in some cases for as long as 2–3 months.

Mortality appeared to have been negligible. While there had been reports of deaths in cases with febrile illness, the etiology of these cases was not clearly determined. There was, however, a report of at least one death in a case with fever and haemorrhage.

RESULTS

Virus isolation from acute sera

Altogether 66 sera were collected in the acute stage of the illness. A total of 60 sera were inoculated—59 into mice and 57 into tissue culture (56 sera were inoculated into mice and into tissue culture) (Table 1). Virus isolations were obtained from the sera of 26 cases—from 21 cases in mice and in tissue culture, from 2 cases in mice only, and from another 3 in tissue culture only.

Reisolation of virus was successful from all the 20 cases in which it was attempted in mice and/or tissue culture (in 16 cases, virus was reisolated both from mice and from tissue culture). In each case the virus was identified by testing either the primary isolate or the reisolate (and frequently both) in QCF tests.

Of the 26 virus strains isolated, 23 were identified as CHIK, 2 as Chandipura (CHP), and 1 as DEN-4. The identity of the DEN-4 isolate (strain No. 653705) was also confirmed by neutralization tests in MKTC. The isolation of the two CHP virus strains has been reported by Bhatt & Rodrigues (1967).

Tissue culture isolation and reisolation of CHIK virus strains was possible in BS-C-1 cells.

The DEN-4 strain (653705) was isolated and reisolated in tissue culture only; the presence of the virus was detected through resistance to polio challenge in MKTC by the interference method described by Paul & Banerjee (1965); the original inoculations were made in BS-C-1 or BHK-21 cells.

Table 1. Isolation of chikungunya virus from human sera

Collection of sera (days after onset)	No. of strains isolated/ no. of sera tested		Total
	In mice	In tissue culture	
1	1/2	1/2	1/2 [a]
2	7/17	7/17	7/17 [b]
3	12/18	11/19	12/19
4	2/7	2/6	3/7 [c]
5–8	0/15	0/13	0/15
total	22/59	21/57	23/60

[a] Chandipura virus was isolated from the second serum in tissue culture.

[b] In addition to the 7 chikungunya isolates, 1 isolation of Chandipura virus and 1 isolation of dengue type 4 virus were obtained, the former being isolated both in mice and in tissue culture and the latter in tissue culture only.

[c] One strain of chikungunya virus was isolated in mice only, another strain in tissue culture only, and the third strain both in mice and tissue culture.

There appeared to be very little difference in the CHIK virus isolation rate from sera obtained on the second, third, or fourth day of illness (Table 1). No virus was isolated from sera obtained later.

Results of mouse cross-challenge test

No resistance to cross-challenge was noticed in any of the mouse groups inoculated with sera from the 31 patients in whom this test was satisfactorily carried out (survival ratios in all cases were lower than 30%). No virus had been isolated from any of the 31 cases. The surviving mice from the groups inoculated with serum No. 653705 were discarded too soon after the challenge for a satisfactory interpretation of the results to be made.

Titre of CHIK virus in acute human sera

When 6 of the 8 sera assayed for virus content were titrated simultaneously in infant mice and in BS-C-1 cells, the titres of virus detected were consistently lower in BS-C-1 cells (Table 2).

The titre of the virus was highest—$10^{6.5}$ infant mouse LD_{50} of virus per 1.0 ml of serum—in a serum specimen obtained on day 3 of illness. Lower titres of virus were detected in the other four sera collected on day 3 of illness ($10^{2.7}$–$10^{4.7}$ LD_{50}). Titres of $10^{5.5}LD_{50}$–$10^{5.7}LD_{50}$ of virus were obtained in sera

Table 2. Titre of chikungunya virus in human sera

Specimen no.	Age/sex	Date of collection (in 1965)	No. of days after onset of illness	Titration in infant mice		Titration in tissue culture (BS-C-1)	
				Date of titration (in 1965)	Titre [a]	Date of titration (in 1965)	Titre [a]
653487	7/M	9 June	1	28 June	5.5	28 June	3.4
653492	25/M	10 June	2	28 June	5.7	28 June	4.7
653904	14/M	21 June	2	30 June	5.7	30 June	3.3
653493	35/F	10 June	3	29 June	4.5	ND	ND
653494	20/F	10 June	3	29 June	4.7	29 June	2.7
653496	34/M	10 June	3	29 June	2.7	ND	ND
653902	17/F	19 June	3	30 June	3.2	30 June	±2.6
653903	28/F	21 June	3	30 June	6.5	30 June	±3.3

[a] The figures represent the logarithm of the LD_{50} or $TCID_{50}$ titres, in infant mice and BS-C-1 cells, respectively, per 1.0 ml of serum: ND indicates that the titration was not done.

collected on day 1 and day 2 of illness. There appeared to be no relation between the age of the individual and the titre of the virus.

Results of CF tests

Paired samples of acute–convalescent sera were obtained from 48 patients, including all those from whom CHIK, DEN-4, and CHP viruses had been isolated. The detailed results of serological tests with 8 paired sera that are representative of the results obtained are shown in Table 3.

All the 23 patients from whom CHIK virus had been isolated as well as 9 of the remaining 25 patients showed either conversion from negative to positive or a 4-fold or higher rise in titre of CF antibodies to CHIK virus (Table 3, represented by sera no. 1, 2, and 5). An additional six patients (represented by sera no. 3, 6, and 7) had antibodies to CHIK virus in both serum samples in titres of 1 : 32–$\geqslant$ 1 : 64, probably indicating that they had been recently infected.

The patient (no. 4) who yielded a DEN-4 isolate (653705) had an 8-fold or higher rise in titre of CF antibodies to group B arboviruses. Three other patients (represented by serum no. 5) had 4-fold or higher rises in titre of CF antibodies to group B arboviruses. CF antibodies to group B arboviruses having similar titres in both serum samples were detected in 30 of the remaining 44 patients (represented by sera no. 2, 3, 6, 7, and 8). However, the titre of CF antibodies did not exceed 1 : 8 in the sera of 20 out of these 30 patients.

The serological response to CHP virus infection of the two patients (no. 7 and 8) from whom CHP virus was isolated has been described in a previous report (Bhatt & Rodrigues, 1967).

In three patients no antibodies were detected in either serum sample to any of the antigens tested. Antibody to SIN virus was detected (titre 1 : 4) in only one patient.

Single convalescent sera

A total of 88 sera from patients seen only in the convalescent stage of illness were tested for CF antibodies to JE, DEN-1, DEN-2, KFD, SIN, and CHIK viruses. Antibodies to group B arboviruses were detected in 80 sera (91%); 34 sera (39%) had antibody titres exceeding 1 : 8, and of these 14 exceeded 1 : 16. Antibodies to CHIK virus were detected in 80 sera (91%) and titres exceeding 1 : 16 were detected in 65 sera (74%). Low titre antibodies to SIN virus (1 : 4) were detected in only two sera, both of which, however, had antibody titres of $\geqslant$ 1 : 64 for CHIK virus.

Sera from contacts

The 37 sera obtained from contacts of febrile cases were tested for CF antibodies to JE, DEN-1, DEN-2, KFD, SIN, and CHIK viruses. Antibodies to group B arboviruses were detected in 26 sera

Table 3. Results of CF tests with paired serum samples

No.	VRC specimen number	Age/ sex	Collection of sera		Virus isolation from acute stage sample [a]	CF titres [b]									Interpretation
			Date (in 1965)	No. of days after onset of illness		JE	WN	DEN-1	DEN-2	DEN-3	DEN-4	KFD	SIN	CHIK	
1	653494-1	20/F	10 June	3	chikungunya	<4	—	<4	<4	—	—	<4	<4	<4	chikungunya
	653494-2		15 July	38		<4	—	<4	<4	—	—	<4	<4	⩾64	
2	653542-1	50/F	5 June	8	NVI	<4	—	4	<4	—	—	<4	<4	4	
	653542-2		11 July	44		<4	—	4	<4	—	—	<4	<4	⩾64	chikungunya
3	653510-1	45/M	10 June	4	NVI	4	—	8	8	—	—	4	<4	32	previous chikungunya
	653510-2		11 July	35		4	—	8	8	—	—	4	<4	32	and group B
4	653705-1	10/M	15 June	2	dengue type 4	4	<4	<4	8	16	4	4	<4	⩾64	dengue type 4 and
	653705-2		13 July	30		256	256	256	128	⩾512	128	32	<4	⩾64	previous chikungunya
5	653497-1	40/M	10 June	2	chikungunya	<4	<4	4	4	4	<4	<4	<4	<4	chikungunya and
	653497-2		15 July	37		8	8	16	16	16	8	UNS	<4	32	group B
6	653484-1	32/M	9 June	8	NVI	8	—	16	32	—	—	16	<4	32	previous chikungunya
	653484-2		12 July	41		8	—	16	32	—	—	16	<4	16	and group B
7	653514-1	35/F	10 June	2	Chandipura	<4	—	<4	4	—	—	<4	<4	32	Chandipura and
	653514-2		17 June	9		<4	—	<4	4	—	—	<4	<4	32	previous chikungunya
	653514-3		11 July	33		<4	—	<4	4	—	—	<4	<4	32	
8	653703-1	45/M	15 June	1	Chandipura	4	—	4	4	—	—	<4	<4	<4	Chandipura and
	653703-2		13 July	29		8	—	8	4	—	—	4	<4	4	previous group B

[a] NVI signifies that no virus was isolated.

[b] A dash indicates that the serum was not tested with these antigens: UNS signifies that the test was unsatisfactory.

Table 4. Virus isolations from *Aedes aegypti* mosquitos

VRC specimen no.	Date of collection (in 1965)	No. of mosquitos in the pool	Virus isolated
653530	14 June	37	dengue type 4
654018-2	1 July	28	chikungunya
654020-1	2 July	50	dengue type 4
654020-2	2 July	44	dengue type 4
654021-1	3 July	50	dengue type 4
654021-2	3 July	23	dengue type 4
654129-1	14 July	50	dengue type 4
654129-2	14 July	50	dengue type 4
654129-3	14 July	23	chikungunya
654174-1	15 July	50	dengue type 4
654174-2	15 July	14	dengue type 4
654175-1	16 July	50	chikungunya
654176-1	17 July	50	chikungunya
654176-2	17 July	33	chikungunya

(70%) and the majority (17 sera) had titres of 1 : 16 or higher. Antibodies to CHIK virus were detected in 15 sera (40%) and eight of these had titres of 1 : 16 or higher. Antibody titres of 1 : 4 in two sera and 1 : 16 in one serum were detected to SIN virus. These sera also had antibody titres of 1 : 8, 1 : 32, and $\geqslant$ 1 : 64, respectively, to CHIK virus.

Virus isolation from Aedes aegypti

Altogether 34 pools containing a total of 1 182 *Aedes aegypti* were tested in mice for the presence of virus: 14 pools, containing 552 mosquitos, yielded 9 strains of DEN-4 virus and 5 strains of CHIK virus (Table 4). These 552 mosquitos had been collected between 14 June and 17 July 1965, and the 14 pools contained from 14 to 50 mosquitos per pool.

DISCUSSION

It is clear from the data that CHIK virus was mainly responsible for the outbreak of febrile illness. The virological and serological findings have shown that in 79% of the patients from whom paired serum samples were obtained, CHIK virus was the probable cause either of the illness under investigation or of an apparently recent infection of the individual. Of the 88 patients from whom sera could be collected only in the convalescent stage of the illness, 74% showed evidence of a probable recent infection with CHIK virus. Confirmatory evidence of the etiological role of CHIK virus was obtained by the isolation of 23 strains of the virus from sera and 5 strains from *Aedes aegypti*. It is probable that the rate of isolation from mosquitos would have been higher if the mosquitos had been collected during April and May, the months of peak incidence of human cases.

It is of interest to refer here to the finding made in a brief serological survey in Nagpur city in 1964 when HI antibody for CHIK virus was detected in only 1 of 50 human sera (Virus Research Centre, Poona, unpublished data). The 1965 epidemic appears to have resulted from a new invasion of the region by the virus.

The isolation of 9 strains of DEN-4 virus from *Aedes aegypti* mosquitos and 1 strain from a human serum is evidence of the fact that DEN-4 virus also played some role in the outbreak. This is confirmed by the serological findings. In addition to the above-mentioned patient who yielded DEN-4 virus, 3 other cases had 4-fold or higher rises in CF antibody titre to group B arboviruses. Evidence of probable recent infection with group B arboviruses was detected in 14 of the 88 unpaired convalescent sera.

A high prevalence of DEN-1 and DEN-2 viruses in Ramtek, Umrer, and Kamptee—all fairly close to Nagpur city—was found by Smithburn et al. (1954) in a preliminary survey in 1952. Results of a subsequent survey by the VRC in 1958 showed that dengue virus was highly endemic in Ramtek, infecting practically all before they reach the age of 6 years (VRC unpublished data).

CHP virus was isolated from two patients. Of 35 paired sera tested in neutralization tests with CHP virus, the sera of these two patients and of one other patient showed conversion (Bhatt & Rodrigues, 1967).

Neutralizing antibodies to CHP virus were detected in 73 of the 108 sera collected in Nagpur in 1957 and 1958 (Bhatt & Rodrigues, 1967).

It appears therefore that the activity of the DEN-4 and CHP viruses can be attributed to the aggravation of an endemic situation: this aggravation could possibly have been caused by an increase in the density of the population of *Aedes aegypti*, a proved vector of the dengue viruses. Transmission studies by Rao et al. (1967) indicated the possibility of *Aedes aegypti* being a vector of CHP virus. Although studies of the population density of *Aedes aegypti* were not carried out, the widespread storage of water

throughout the city during the dry and warm months between March and early June created conditions that were ideal for increased breeding of *Aedes aegypti*.

It would thus appear that three viral agents were involved in varying degree in the Nagpur outbreak, CHIK virus being the main etiological agent with DEN-4 and CHP viruses playing minor roles.

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RÉSUMÉ

ÉTIOLOGIE D'UNE AFFECTION FÉBRILE, À ALLURE ÉPIDÉMIQUE, QUI A SÉVI EN 1965 DANS LA VILLE DE NAGPUR (ETAT DE MAHARASHTRA, INDE)

Durant les mois d'avril, mai et juin 1965, la ville de Nagpur a connu une importante épidémie d'une affection fébrile caractérisée surtout par des douleurs, notamment articulaires.

L'enquête sérologique a débuté en juin. On a prélevé du sérum chez 66 malades à la phase aiguë de l'affection, et dans 48 cas des sérums couplés (phase aiguë-convalescence) ont pu être obtenus. Des sérums ont aussi été recueillis chez 88 convalescents. Les sérums de phase aiguë ont été inoculés par voie intracérébrale au souriceau et, dans la plupart des cas, également à des cultures tissulaires de divers types. On a d'autre part recherché dans les sérums couplés et les sérums de convalescents les anticorps fixant le complément (FC) à l'égard d'une série d'arbovirus.

L'enquête virologique a consisté en la recherche des virus par inoculation intracérébrale au souriceau de matériel obtenu à partir de lots d'*Aedes aegypti* capturés dans les habitations de la zone affectée par l'épidémie.

Vingt-six souches ont été isolées à partir des sérums de phase aiguë: 23 virus chikungunya, 2 virus Chandipura et 1 virus de la dengue type 4. Sur les 48 paires de sérums, 32 montraient une hausse significative des titres d'anticorps FC dirigés contre le virus chikungunya; 5 autres contenaient des anticorps anti-chikungunya à des titres de 1 : 32 à 1 : 64, indice probable d'une infection récente. Dans 4 cas, on notait une hausse marquée des anticorps FC pour les arbovirus du groupe B. Parmi les 88 sérums de convalescents, 80 (91%) renfermaient des anticorps anti-chikungunya et des anticorps dirigés contre les arbovirus du groupe B.

Neuf souches de virus de la dengue type 4 et 5 souches de virus chikungunya ont été isolées à partir de 14 lots de moustiques (renfermant 552 *A. aegypti*) sur un total de 34 lots comptant 1182 *A. aegypti*.

Il semble que 3 virus aient été responsables dans une mesure variable de l'épidémie, le virus chikungunya étant le principal agent étiologique alors que les virus de la dengue type 4 et Chandipura ne jouaient qu'un rôle accessoire. Comparant les résultats de la présente enquête à ceux d'investigations antérieures, les auteurs concluent que l'épidémie est due à l'exacerbation de l'activité des virus, endémiques, de la dengue type 4 et Chandipura et à la pénétration du virus chikungunya dans la région.

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