A review of the use of Vi-tests in tracing typhoid carriers in the population at large leads the author to conclude that their usefulness has not been proved. Furthermore, until more information is available on the natural history of the Vi-phages, it is premature to identify their presence with that of S. typhi.

DETECTION OF TYPHOID CARRIERS

Victor Bokkenheuser, M.D.

ALTHOUGH typhoid fever is well controlled in countries with generally high hygienic standards, it continues to be a menace in areas with large population groups of low economic status. With limited resources the question arises whether the disease is best prevented by allocating available funds to educational and hygienic measures or whether better results are achieved by intensifying the search for chronic carriers of Salmonella typhi. In countries relying mainly on the latter approach, large-scale investigations for typhoid carriers are done particularly among food handlers. As an example it may be mentioned that more than 20,000 serums are submitted annually to this laboratory for Vi-antibody estimation. The nonreactors are considered to be free of S. typhi, while excreta from the reactors are submitted for bacteriological examination. Because of the striking infrequency of recovery of S. typhi from Vi-reactors, the literature has been examined with a view to assessing the value of current methods for the detection of chronic typhoid carriers.

The Significance of the Vi-Test

Reviewing the literature on the Vi-test, Bokkenheuser[5] found that diverse opinions were held on its practical value. By pooling the results of several authors who used bacterial agglutination tests for the detection of Vi-antibodies, it was calculated that the specificity of the test in detecting typhoid carriers increased with the Vi-antibody titer, but for all practical purposes it did not exceed 10 per cent. In other words, more than 90 per cent of the reactors were free from S. typhi. The lack of specificity was not balanced with a high degree of sensitivity. In fact, about 25 per cent of the known carriers of S. typhi showed no agglutination in a serum dilution of 1:10 and would therefore be classed as nonreactors by most laboratories. As pointed out by the author,[5] the pooling of results obtained by similar, but not identical, technics is controversial, which in turn makes the conclusions questionable. In his comments on the paper, Bensted[4] stressed the weakness involved in compiling the figures but kept an open mind regarding the correctness of the results. It is therefore of considerable interest to study a recent report by the Public Health Laboratory Service Working Party headed by Dr. E. S. Anderson.[51] Their material consisted of 67 known typhoid carriers and 343 normal individuals examined for agglutinins to bacterial TVi suspensions (Standards Laboratory, Colindale). The patients' serums, diluted 1:5,

1:10, 1:20, 1:40, 1:80 and 1:200, were titrated in parallel with the Provisional Standard Serum for Vi agglutination. The titer was indicated by the dilution which gave an agglutination equal in degree to the 1:1,400 dilution of the Provisional Standard Serum. The PHLS Working Party[51] found that 71.5 per cent of the carriers and 1.17 per cent of the noncarriers yielded a positive result in the first serum dilution (1:5). Thus, 28.5 per cent of the carriers failed to show up in the bacterial Vi-agglutination test. In an additional study comprising 29 carriers belonging to four different surveys, they reported that 92 per cent had Vi-agglutinins in a dilution of 1:5 or higher. It was not stated, however, whether or not identical technics were employed in the serological investigation of the pooled material. The authors concluded that these results "still make the test a very useful one, assuming that its specificity is adequate." In view of a "reasonable sensitivity," they felt, "a proportion of positive results (1.17 per cent) without ascertainable cause may be encountered."

Accepting the assumption that in England there is about one typhoid carrier per 100,000 population,[51] the specificity of the bacterial Vi-agglutination test is calculable. It is expressed in the equation[5]:

$$\frac{\text{(No. of Vi-positive carriers in the population)} \times 100}{\text{Total no. of Vi-reactors in the population}}$$

Accordingly the specificity of the test employed by the PHLS Working Party[51] is:

$$\frac{71.5 \times 100}{1.17 \times 100{,}000} = 0.07\%$$

or 99.93 per cent of the reactors detected by this method are normal individuals free of infection with S. typhi. Even if the carrier rate in England were ten times higher than assumed by the PHLS Working Party,[51] 99.3 per cent of the reactors would be noncarriers. These figures are in agreement with the results calculated by pooling the material from different surveys,[5] but do not justify the opinion that the bacterial Vi-agglutination test is satisfactory for the detection of chronic carriers of S. typhi in the population at large. However, it may have limited usefulness in closed groups of individuals among whom a carrier is known to exist.[5]

The PHLS Working Party[51] also gave the bacteriological findings in 29 chronic typhoid carriers of whom 20 were middle-aged, mentally ill females. They found that a single bacteriological examination of excreta was unreliable for the demonstration of S. typhi, but that the examination of three specimens collected at weekly intervals revealed from 70 to 80 per cent of the carriers in their material. From a group of 70 chronic carriers without any particulars as to sex, age, physical or mental state, Schubert, Edwards, and Ramsey[56] isolated S. typhi in 55 cases (78 per cent) on a single examination. Studying 12 chronic fecal carriers Thomson[67] found that the individual excretion rate of S. typhi ranged from 45 x 10^6 to less than 500 organisms per gram of fecal material. While the heavy excreters consistently yielded positive results, S. typhi was recovered rarely from the light excreters. For the purpose of this paper it would have been valuable had the intensity of excretion of S. typhi been correlated with the level of circulating Vi-agglutinins. To the knowledge of the reviewer such information is not available. Thus, at the moment the only permissible conclusions regarding the bacterial Vi-agglutination test are: (1) Based on the results of the Vi-test a population may be divided into reactors, embracing from 2 to 10 per cent of the group[5,51] and nonreactors (90-98 per cent). (2) Some 70 to 80 per cent of the carriers with excretion rates corresponding to those prevalent in the material of the PHLS Working Party[51]

and of Schubert, Edwards, and Ramsey[56] occur in the reactor group. But for each carrier among the reactors there are 100-1,000 normal individuals. (3) The nonreactors include some 20 to 30 per cent of the chronic carriers of the above quoted type. (4) We are ignorant both of the relationship between intensity of excretion of S. typhi and Vi-agglutinin titer and of the exact cause of Vi-agglutinins in noncarriers.

Vi-Hemagglutination—Using human Type O erythrocytes modified with extract of TViI (Bhatnagar), Staack and Spaun[62] found 87.8 per cent sero-reactors (dilution 1:5) among 49 chronic typhoid carriers and 7.4 per cent reactors among 243 allegedly normal individuals. Landy and Lamb,[37] modifying human Type O erythrocytes with a "purified" Vi-antigen derived from E. coli 5396/38, reported 95 per cent sero-reactors (dilution 1:7.5) among 20 confirmed carriers of S. typhi and 1.6 per cent reactors among 252 normal individuals. In regard to sensitivity and specificity, therefore, neither of the methods were distinctly superior to the bacterial Vi-agglutination test.[51]

Better results were obtained by Schubert, Edwards, and Ramsey,[56] who modified sheep erythrocytes with an extract of the Vi-Ballerup strain. Of 179 chronic carriers, 92.2 per cent yielded positive Vi-hemagglutination reaction (dilution 1:10), while not a single reactor occurred among 193 normal individuals. This, although promising, could not be confirmed by Anderson.[3] He found, as did Schubert, Edwards, and Ramsey[56] that many human serums contained agglutinins to sheep erythrocytes and therefore required absorption. Changing to human Type O erythrocytes modified with Vi-Ballerup extract he observed 20 per cent reactors among noncarriers. Since absorption of these serums with TViI suspension did not remove the agglutinins to the modified erythrocytes, he concluded that the agglutinins in the reactive serums from the noncarriers were directed against an erythrocyte attachable component in the Ballerup extract other than the Vi-antigen. Anderson's conclusion does not necessarily hold for an indicator system based on sheep erythrocytes. It has been shown, for example, that both human and alligator erythrocytes modified with Vi-antigen are agglutinable in Vi-antiserum. If such erythrocytes instead are treated with typhoid-O antigen, only the human cells are agglutinable in typhoid-O antiserum.[6]

Thus, at present, there is no evidence to suggest that hemagglutination tests are better than the bacterial Vi-agglutination reaction in the detection of Vi-antibodies associated with the carrier state.

Investigation for S. Typhi

The Chronic Carrier

A chronic typhoid carrier is usually defined as a person from whom S. typhi has been isolated over a period of at least one year.[53] The organisms are usually found in the stools, but the occasional occurrence of true urinary carriers[7,28,39] demands the examination of both types of excreta. Before collecting a fecal specimen some workers recommend the administration of a purgative,[7,57,68] preferably in combination with a drug that causes contraction of the gallbladder.[68] The number of specimens which should be examined to exclude the carrier state is a problem of perennial discussion. It is well known that from some carriers S. typhi is easily recovered, while others only yield an occasional positive specimen.[28,33,39,45,51,57,67] The latter type of carrier is often referred to as an intermittent excreter,[7,39] but without quantitative evaluation of their excretion rates it might well be argued that some of them are permanent light excreters difficult to detect by ordinary bacteriological technics. That this type of carrier requires re-

peated bacteriological examinations was illustrated by the PHLS Working Party,[51] although their results were obtained by combining the findings of four surveys in which technics may have differed. The combined material suggests that a considerable proportion of carriers would have been missed if only one specimen had been examined from each individual. The authors conclude that 21/29 (72 per cent) of the carriers in their material had a better than even chance of being revealed by the bacteriological examination of three specimens of feces; but to exclude the carrier state Rindge[53] insisted on two series of bacteriological investigations with a month's interval. Each series consisted of one duodenal, three fecal, and three urinary specimens. Humbert[28] advocated a 24-months' observation period with 20 negative urinary and fecal specimens followed by three negative duodenal investigations.

Two considerations are paramount in planning to detect typhoid carriers by systematic bacteriological examination of large population groups. In the first place the prevalence of chronic carriers is usually low. Ames and Robins[1] calculated that there was one carrier to 2,500 noncarriers in New York, 1940. They quoted the carrier rate in Missouri as 1:3,500, but pointed out that these were minimum figures. The PHLS Working Party[51] estimated the carrier rate in England in 1961 to be as low as 1:100,000. Second, since many carriers are intermittent or light excreters, some of them will remain undetected by reasonable routine investigations involving for example the examination of three fecal and three urinary specimens per individual.[67] Thus, the systematic, bacteriological search for carriers in unsuspected population groups is bound to be both unrewarding and costly.

Transportation of Specimens

If bacteriological examination of excreta is expected to be delayed for more than two to four hours, special precautions should be taken to ensure the survival of the pathogens.[25,55] By inhibiting the multiplication of bacteria and possibly by inactivating the bacteriophages,[65] Sachs's buffered glycerol saline with indicator[55] has proved a useful transportation medium[25] for a period not exceeding 48 hours.[43,65] Beyond this its slight bactericidal effect substantially reduces the number of surviving bacteria.[65] The incorporation of formalin to a final concentration of 1:10,000 is disadvantageous because it increases the death rate of S. typhi and is without appreciable effect on bacteriophages.[65] An interesting modification of buffered glycerol saline was suggested by Huet and Bonnefous.[27] Stored in their transport medium for six days at 30° C, half of the Salmonella-containing stools still yielded a growth of Salmonella, and if stored at 4° C half of the specimens remained positive for over a month. Information of the medium's capacity to preserve S. typhi, particularly under controlled field conditions, is desirable.

Strangely enough, Stuart's transport medium[64] has not been recommended for the investigation of Salmonella infections. In view of its successful application to a long-range of delicate organisms including Shigella sonnei, it would be reasonable to expect that it might prove useful also for the survival of S. typhi.

Attempting to simplify methods of preserving pathogens in stools, Lie Kian Joe, et al.,[42] suggested drying a large drop (0.2 ml) of fecal suspension on filter paper. To impregnate the filter paper with a small drop, to soak it in fecal fluid or to allow more than three hours for the dehydration were considered inferior technics.[30] Upon arrival in the laboratory the impregnated paper was soaked in saline for 15 minutes and the suspension was then inoculated heavily on differential plates. Alternatively, the dehydrated specimen was placed in

broth, macerated, and subcultured. The number of positive results from the dehydrated specimen after five days' storage was only 17 per cent lower than the results obtained by direct, immediate plating of the original specimen.[42] Unfortunately, enrichment media were not used. Since nonpathogens often resisted dehydration poorly, cultivation of the material not infrequently resulted in a comparatively pure growth of pathogens ("relative enrichment").

The main objection to employing the filter paper technic in searching for S. typhi, it was felt, was the enrichment procedures requiring heavy inoculation. This assumption is probably erroneous. In the first place, it is generally not appreciated that 0.2 ml (one drop) of fecal suspension is the recommended amount for filter paper impregnation.[30] Furthermore, five drops could easily be prepared from each specimen giving a sample of 1 ml, sufficient for currently used enrichment procedures. Second, a very attractive, water-proof disposable cardboard outfit was devised by Megay, Marek, and Grims[44] for drying stool specimens. It allowed for transportation of 1-2 ml of fecal material and gave very promising preliminary results. Lastly, several authors have noted that better results are often obtained if the fecal suspension is diluted 1:100-1:1,000 prior to the inoculation of enrichment media.[52,60] Thus, in the absence of documented objections to the use of dehydrated fecal specimens, it seems well worth while to pursue the investigation.

Enrichment Media

Most bacteriological laboratories have adopted enrichment procedures in their search for S. typhi. Of the available media,[61] tetrathionate-brilliant green-bile broth[31] seems most beneficial for the growth of Salmonella in general[10,11,25,32,52] and selenite-F broth for the detection of S. typhi[10,23,24,25,32] in particular. Some authors have found selenite-F broth satisfactory for Salmonella isolation in general.[61] Attributing the selectivity of the medium to the formation of selenopolythionates or other selenosulfur compounds rather than to free selenium, Smith[59] suggested that they interfere with the sulfur metabolism by acting "as growth-inhibitory analogues of highly preferred sulfur sources." While for maximum efficiency it seems advisable to use a peptone of known quality and to check the phosphate content of the medium,[50] the addition of cystine[50] appears to be of questionable value. Although it will enhance the growth of many pathogens,[59,60] it may lead to a reduced selectivity.[59] Incorporating about 0.5 per cent fermentable carbohydrate is undoubtedly beneficial,[40,59] but whether lactose or mannitol is preferable is still a moot point.[24,40,59,60,61] In this connection it would be of considerable importance to determine the possible influence of glycerol from the transport medium on the selectivity of selenite-F broth. The usual proportion of 1 ml of transport medium to 9-10 ml of selenite-F broth results in a mixture containing about 3 per cent glycerol which is six times the prescribed amount of fermentable carbohydrate.

An attractive modification of selenite-F broth was suggested by Stokes and Osborne[63] who added small amounts of brilliant green and sodium taurocholate to the medium. Tested with pure cultures it yielded encouraging results, but, to the knowledge of the revewer, it has not yet been submitted to critical trials.

As mentioned above, the selectivity of selenite-F broth may in certain cases depend upon the size of the inoculum. It is often recommended to use one part of material to be examined to 9-10 parts of medium.[40,66] Other workers, however, have reported better results with inocula about 100 times smaller.[52] To explain this the following suggestions have been advanced: (a) the larger

inoculum may contain substances toxic for the pathogens, the effect being abolished by dilution[46]; (b) when the absolute number of nonpathogens is excessive they may outgrow the pathogens.[52] The latter concept presupposes a bacterial competition for essential metabolites or the development of bacteriostatic or bactericidal compounds by nonpathogens. A faster growth rate of nonpathogens does not adequately explain the easier isolation of pathogens from small inocula.

Considering on one hand the possibility of a small number of pathogens in a specimen, and on the other the presence of toxic substances or of nonpathogens either competing for essential metabolites or producing bacteriostatic compounds, it would appear advantageous to inoculate enrichment media both heavily and lightly from the same specimen.

Isolation Media

In order of popularity the most favored media for the isolation of S. typhi are Wilson and Blair's agar, desoxycholate-citrate agar[42,43] and SS agar. Wilson and Blair's medium either in its original form[70] or in one of its modifications[9,18,35] is considered the most selective medium,[10,18,24,25,35] but for several reasons many laboratories have been reluctant to use it. First, the difficulties in its preparation and the variation from batch to batch in its growth-promoting power demand constant and careful attention.[24,46] Second, some strains of S. typhi have failed to produce characteristic colonies,[22,35] while the blackening of others may be retarded so that a 48-hours' incubation period is required before a culture may be discarded as negative.[9,24] Third, on crowded plates the colonial morphology of S. typhi is atypical.[34,48] And fourth, on storage the selective property of the medium declines rapidly.[9,24] These drawbacks may be overcome to a large extent by (a) employing commercially available dehydrated Wilson and Blair's medium[24]; (b) increasing the concentration of ferrous sulfate[22]; and (c) by inoculating plates with progressive dilutions of each specimen.[34,48] The desoxycholate-agar yields from 2 per cent to 15 per cent less positive results than the Wilson and Blair's medium,[42-44] but is easy to prepare, reproducible, stable, and allows the colonies to develop within 24 hours. Similar qualities are found in the SS plate[42,43] but some authors have reported this medium to yield about 50 per cent less S. typhi than the Wilson and Blair's medium.[11] However, there is general agreement that no single medium is capable of yielding a growth of S. typhi from all infected samples and that the number of positive results increases with the number of media employed.

For subcultures from selenite-F broth Wilson and Blair's medium is again superior with a 7.5 per cent higher yield than the desoxycholate-citrate agar.[23] It has been reported, however, that some strains of S. typhi develop poorly in enrichment media.[46,52]

Sewage

In his excellent review on the isolation of S. typhi from sewage, Robinson[54] emphasized the valuable improvement introduced by Moore's continuous sampling technic.[47,48] The method was further refined[54] by substituting gauze pads[47,48] for Higgins' calcium alginate wool,[21] which is soluble in the nonbactericidal sodium hexametaphosphate. Robinson[54] did not establish, however, that the better yield of S. typhi from the calcium alginate swabs was attributable entirely to the alginate dissolving action of sodium hexametaphosphate. It is feasible that the compound, in addition, might have, for example, a direct disaggregating effect on the sewage particles.

Pads should be submerged in the moving sewage for at least 48 hours.[19,34,47,

[54] To speed up and facilitate investigations, Harvey and Phillips[19] introduced a "wipe-swab," which collects the sample by being passed along the side of the sewer. It yielded cultures of great purity[19] and in combination with the "left-down" pads it appeared particularly useful in distinguishing between a recent or a long-standing contamination of the sewage. It should be mentioned that S. typhi may survive in sewage for at least two weeks and other Salmonellae considerably longer.[58] With the development of the water-closet swab for continuous sampling[36] it is now possible to trace the infection from the infected sewers through lateral branches to the excreter's closet.

From comparative studies it appears that the Wilson and Blair agar and the selenite-F broth form the best combination for the recovery of S. typhi from sewage.[36,48,54] Since certain sewage organisms may overgrow the S. typhi in the selenite-F broth, a primary plate is necessary.[48] To avoid crowded growth resulting in atypical unrecognizable colonies, the specimens should be serially diluted with planting of aliquots from each suspension. This applies both to primary cultures and subcultures from the selenite-F broth.[48,54]

In regard to the planting in selenite-F broth, some workers prefer a large inoculum[12,36,54] and others a small one.[19,48] In this context it may be noted that Leifson[40] found a retarded growth rate of S. typhi in selenite-F broth in the presence of tremendous excess of E. coli; that Rappaport, et al.,[52] obtained better results by planting the enrichment medium with diluted material; and that S. typhi sometimes is isolated from the primary plates only.[46,48] Because of this it would probably be advantageous to inoculate portions of selenite-F broth not only with the original suspension but also with decimal dilutions.

Robinson[54] showed that the optimum incubation period for sewage in selenite-F broth was 6-12 hours and that the yield of S. typhi declined rapidly with incubation beyond 18 hours. Still better results might be obtained by serial enrichment procedures,[20] in which aliquots of enrichment culture, after appropriate incubation, are transferred to fresh portions of enrichment medium. Subcultures onto Wilson and Blair's agar should be made from each enrichment culture within 18 hours of incubation.

A negative result of sewage examination is no guarantee for the absence of S. typhi. It may be attributed to one or more of the following causes[49]: (a) intermittent excretion by the infected individual; (b) contamination of the sewage with disinfectant; (c) high dilution of the typhoid organisms as may occur in the main sewers from large populations; and (d) sampling or laboratory technics employed.

Investigation for Bacteriophages

Impressed by the host specificity of bacteriophages, Guélin and le Bris[13] suggested that the demonstration of typhoid phages in water might provide evidence of contamination with S. typhi. Technically it requires a bacterial indicator strain, rich in Vi-antigen, susceptible to the largest possible number of typhoid Vi-phages and resistant to most, if not all, other phages. Several useful strains are available,[2,8,15,38,41] but since none of them is lysed by all phages, better results are obtained by employing a range of indicator organisms.[15,16,38] Their tendency to lose specificity in the laboratory necessitates periodical checking.[16]

The phages may be demonstrated directly or after enrichment of aliquots of the sample in young liquid cultures of the indicator strain.[8,15] In both cases the phage suspensions must be freed of vegetative bacteria either by filtration,[8] by exposure to 56° C for 30 minutes,[15] or by the action of detergents.[29] A

small volume of the ensuing suspension is then placed on a lawn of the indicator strain, which is incubated and observed for lysis. Although the Vi-phages are characterized by the production of large plaques,[16,17] the phages should be obtained in pure culture by serial propagation on susceptible strains. The specificity of the pure suspension is demonstrated by its inability to lyse any other strain but Vi-carrying S. typhi.[38,41] This may be supplemented by phage antiserum neutralization tests.[41]

The demonstration of typhoid Vi-phages in sewage and water is often claimed to be easier than the detection of S. typhi. This is attributed to the large number of phages in the stools of infected individuals,[8] to the better survival of bacteriophages in nature,[14,15] and to technical procedures which, in search of phages, remain unaffected by the large number of saprophytes present in some of the samples.[8]

The crucial point in this approach to the detection of typhoid carriers hinges on the true significance of the presence of typhoid Vi-phages. Some workers[8,17,38] consider these phages so intimately linked with S. typhi that their demonstration for all practical purposes may be taken as proof of the presence of S. typhi. In addition, it has been suggested that a high Vi-phage content is evidence of recent contamination, while the isolation of a few phages would indicate an earlier pollution.[17] Guélin[15] observed that the number of typhoid Vi-phages in a specimen was proportional to its content of fecal E. coli, Type I. In other words the presence of Vi-phages would be indicative of fecal pollution. Carrying the investigation a step further, Hudemann[26] found that Vi-phages were absent in stools from a variety of animals and suggested that they might indicate fecal contamination of human origin. In addition, he showed that only 6.7 per cent of typhoid patients and carriers excreted Vi-phages and that they could be demonstrated in 2 per cent to 4 per cent of normal individuals, in patients with diarrhea of unknown etiology, and in children with infantile diarrhea. Furthermore he recovered the Vi-phages from vegetables (13 per cent to 48 per cent), from meat products (6 per cent to 13 per cent), and raw milk and products thereof (33 per cent). It is noteworthy that, despite careful investigation, only the typhoid patients and carriers yielded a growth of S. typhi. In Hudemann's[26] opinion the presence of phages did not necessarily indicate the presence of S. typhi, since the possibility exists that the typhoid Vi-phages might be able to propagate in Vi-carrying nontyphoid organisms. This view is supported by Anderson[2] and Valeriu[69] who state that "the possibility of typhoid Vi-phage multiplying in heterologous strains reduces the importance which most workers attribute to its presence in polluted water."

In conclusion, therefore, it appears that until more information is available on the natural history of Vi-phages, it is premature to identify their presence with that of S. typhi.

Conclusions

The usefulness of the Vi-tests in tracing typhoid carriers in the population at large has not been proved.

The isolation of S. typhi from man requires careful planning both with regard to the preparation of the patient, to the circumstances of transportation of the specimens, and to the selection and control of laboratory technics. Because of the low carrier rate and the intermittence in excretion, the cost of systematic search for carriers in large population groups can hardly be considered rewarding either from a preventive or from an economic point of view.

The isolation of S. typhi from sewage has been greatly improved by the introduction of modern sampling technics with

gauze pads, calcium alginate wool pads, or "wipe-swabs." Although the laboratory procedures have been worked out in some detail, much additional research is required to establish the optimum conditions for the isolation of S. typhi.

The demonstration of typhoid Vi-phages in sewage and water appears to be indicative of fecal contamination of human origin. Lack of ecological information on these phages prevents further conclusions.

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