BACTERIAL FLORA OF THE GASTROINTESTINAL TRACT IN SOUTHERN INDIAN CONTROL SUBJECTS AND PATIENTS WITH TROPICAL SPRUE


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The viable bacterial flora of the gastrointestinal tract has been studied by peroral intubation in 12 control southern Indian subjects and in 33 patients with tropical sprue. Eight of the control subjects had viable bacteria in the upper jejunum in concentrations of 10⁶ to 10⁷ per ml, and all had organisms throughout the ileum (mean count 10⁶ per ml). Patients with tropical sprue had similar flora in the small intestine, there being no statistically significant differences between the patients and the controls. In the patient group significantly more of those with a convoluted jejunal mucosa had enterobacteria and streptococci in the upper jejunum than those with leaf-shaped villi, and more of those with an abnormal barium meal had enterobacteria than those with normal X-ray findings. The individuals with sprue had significantly more enterobacteria and veillonelae in the stools than did the controls. In patients with a history of more than 1-year duration, the total number of aerobes in the stool was greater than the total number of anaerobes. There was no relationship between the bacteriological findings and the course of the disease or response to therapy. The presence of bacteria in the small intestinal lumen of some patients with sprue appears to be a nonspecific finding and the relationship of these bacteria to the malabsorption of various substances is obscure and needs further investigation.

For many years it has been postulated that tropical sprue may be an infective disease and the response of some cases to antimicrobial therapy suggests that intestinal bacteria may play a role in its pathogenesis. Search for known bacterial pathogens by culture of feces, even in epidemics of sprue, has been unrewarding. Studies of bacteria in the lumen of the intestine have been carried out in relatively few subjects, in some cases without anaerobic techniques, and with varying results.

This study was therefore undertaken to quantify the different types of aerobic enabling Dr. Bhat to gain experience in anaerobic bacteriology and for their help and advice. They also wish to thank Dr. S. Gorbach for his help and encouragement; Miss H. Isaac, Mr. R. T. Swaer, Mr. E. Samuel, and Mrs. N. Dornipandian for valuable technical help; Mr. G. Juddiam and Mr. S. Mathews for statistical help; Mr. J. Piahihakan for artistic and photographic assistance; and Mr. S. Joel for secretarial assistance.

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and anaerobic bacterial flora of the gastrointestinal tract in patients with tropical sprue and in control subjects.

**Material and Methods**

Thirty-three patients with tropical sprue (32 southern Indians and one American) and 12 apparently healthy asymptomatic control subjects were studied. The Indian patients and the controls all belonged to the poorer socioeconomic group and had a largely vegetarian diet which was similar to that described by Rao and Rao. The American, a male Peace Corps volunteer, ate a good diet containing a large amount of animal protein. All patients and controls were admitted in a metabolic ward and were given a standard diet containing 50 to 60 g of fat per day. Full gastrointestinal and hematological studies were carried out as described previously. As soon as possible after admission, the subjects, in the fasting state, were intubated with a sterile radiopaque double-lumen polyvinyl tube with a balloon and small mercury bag attached. As the tube progressed down the intestine, specimens were aspirated from the stomach, the first loop of jejunum just beyond the ligament of Treitz (70 to 110 cm from the incisor teeth), from the lower jejunum (140 to 200 cm), from the upper ileum (220 to 250 cm), and from the lower ileum (260 to 315 cm). At each level the first aspirate was discarded to minimize contamination from any residual juice remaining in the tube from the previous level. Specimens were collected on ice and transferred immediately to the laboratory for processing. When intubation was prolonged, fluids were given by mouth after the lower jejunal sample had been obtained. Samples of saliva and a fresh specimen of stool were also obtained from each patient and were processed immediately.

Specimens were diluted in 10-fold steps from $10^{-1}$ to $10^{-7}$ with normal saline. Each dilution (0.1 ml) was spread on plates containing the various media listed in table 1. For the anaerobic studies, extensive precautions were taken to minimize exposure to air. Specimens were inoculated as rapidly as possible and placed immediately in an anaerobic jar fitted with a cold catalyst and continuously flushed with nitrogen. Steel wool soaked with acidified copper sulfate solution was placed in the jar, which was then closed, evacuated, filled with hydrogen, reevacuated, and finally filled with 90% hydrogen and 10% carbon dioxide. The anaerobic plates were incubated at 37 C for 5 days. Microaerophilic incubation was performed using anaerobic jars without addition of steel wool or cold catalyst. The jars were partially evacuated and refilled with 10% CO$_2$ and incubated at 37 C for 3 days. The aerobic cultures were incubated at 37 C for 1 to 2 days.

The following groups of organisms were identified by standard bacteriological techniques: enterobacteria, aerobic streptococci other than enterococci (henceforth termed streptococci), enterococci, neisseriae, staphylococci, lactobacilli, yeasts and yeastlike organisms, fusobacteria including sphaerobacter, bacteroides including Bacteroides melaninogenicus, bifidobacteria, veillonellae, and lecithinase-positive clostridia, mainly Clostridium welchii. Surface viable counts were done by the spreading method. Results were expressed as the number of viable organisms per milliliter of intestinal juice or per gram of feces.

The results of the various investigations and the bacteriological findings at all levels were entered on International Computers and Teleprinters punch cards to facilitate data analysis and the detection of possible correlations.

**Results**

The control subjects all excreted less than 6 g of fat per day in the stool, had normal vitamin B$_{12}$ absorption, normal barium meal findings, and 7 out of 12 had a normal xylose excretion. The jejunal biopsy was normal by Western standards in 2 and in the remainder showed minor changes, such as increase in the depth of the glandular layer and increased cellular infiltration of the lamina propria, which occur commonly in normal southern Indian subjects.

The patients with sprue all had steatorrhea (mean, 18.0 g; range, 7 to 33 g of fat per day) and xylose malabsorption (mean, 8.6%; range, 1 to 23% excretion in 5 hr) and 22 had malabsorption of vitamin B$_{12}$. The jejunal biopsy was abnormal in all; 13 had a leaflike villous pattern, 20 a convoluted pattern, and histologically 7 had mild changes, 12 had partial villus atrophy, and 13 severe partial villous atrophy.

In the saliva the pattern of bacterial flora was similar in both the control and patient groups, the predominant organisms being neisseriae (mean count $10^7$ per ml), fusobacteria ($10^6$ per ml), streptococci ($10^7$ per ml), veillonellae ($10^6$ per ml), and bacteroides ($10^6$ per ml).
Table 1. Culture Media

<table>
<thead>
<tr>
<th>Medium</th>
<th>Organism</th>
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<tbody>
<tr>
<td>Sheep blood agar with neomycin (25 µg/ml)</td>
<td>Fusoacteria, including sphaerosporus</td>
</tr>
<tr>
<td>Ethyl violet agar&lt;sup&gt;8&lt;/sup&gt;</td>
<td>Bacteroides</td>
</tr>
<tr>
<td>Rogosa SL agar&lt;sup&gt;8&lt;/sup&gt;</td>
<td>Bacteroides melaninogenicus</td>
</tr>
<tr>
<td>Reinforced clostridial agar&lt;sup&gt;4&lt;/sup&gt; with neomycin (25 µg/ml)</td>
<td>Fusoacteria, including sphaerosporus</td>
</tr>
<tr>
<td>Egg-yolk agar with neomycin (100 µg/ml)&lt;sup&gt;21&lt;/sup&gt;</td>
<td>Bifidobacteria</td>
</tr>
<tr>
<td>Veillonella agar&lt;sup&gt;2&lt;/sup&gt; with vancomycin</td>
<td>Bifidobacteria</td>
</tr>
<tr>
<td>Microaerophilic organisms</td>
<td>Lecithinase-positive clostridia</td>
</tr>
<tr>
<td>Rogosa SL agar&lt;sup&gt;4&lt;/sup&gt;</td>
<td>Veillonella</td>
</tr>
<tr>
<td>Aerobic organisms</td>
<td>Lactobacilli</td>
</tr>
<tr>
<td>MacConkey agar&lt;sup&gt;12&lt;/sup&gt;</td>
<td>Enterobacteria</td>
</tr>
<tr>
<td>Sheep blood agar&lt;sup&gt;19&lt;/sup&gt;</td>
<td>Other aerobic Gram-negative rods, e.g., Pseudomonas, etc.</td>
</tr>
<tr>
<td>Sheep blood agar with neomycin (25 µg/ml)</td>
<td>Enterococci</td>
</tr>
<tr>
<td>Mannitol salt agar&lt;sup&gt;17&lt;/sup&gt;</td>
<td>Streptococci (including enterococci)</td>
</tr>
<tr>
<td>Sabouraud dextrose agar&lt;sup&gt;6&lt;/sup&gt; with neomycin (350 µg/ml)</td>
<td>Staphylococci</td>
</tr>
<tr>
<td></td>
<td>Micrococcis</td>
</tr>
<tr>
<td></td>
<td>Neisseriae</td>
</tr>
<tr>
<td></td>
<td>Streptococci (including enterococci)</td>
</tr>
<tr>
<td></td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td></td>
<td>Yeasts</td>
</tr>
</tbody>
</table>

<sup>8</sup> Purchased as dehydrated media from the Difco Laboratories, Detroit, Michigan.
<sup>12</sup> Purchased as dehydrated media from Oxoid, Ltd., London, England. Other media were prepared in the laboratory.

In the fasting gastric juice organisms were recovered in half of the controls and from 23 (68%) of the patients with sprue. These organisms were generally similar to those in the saliva, although two controls and 14 patients also had enterobacteria (fig. 1). Statistically, there was no significant difference between the two groups when either total flora or individual organisms were considered. There was also no correlation between the results of the augmented histamine test and the number of type of organisms.

In the upper jejunum (fig. 1) there were no viable organisms in 4 of the controls and 4 of the patients. The oral type flora, such as fusobacteria and nonenterococcal streptococci, were present in smaller numbers than in the gastric juice. Half the control subjects and two-thirds of the sprue patients had enterobacteria at this level. The mean log count of enterobacteria in the patients (4.5) was slightly higher than in the controls (3.7). However, none of these differences attain statistical significance.

In the lower jejunum and upper and lower ileum (fig. 1), the number of individuals from whom oral type flora were isolated decreased and the number of individuals from whom enterobacteria and enterococci were isolated increased. The mean counts of these latter two groups of organisms also increased in the more distal regions. In no case was there any significant difference between the control and the patient group.

In the stools (fig. 1) the total number of viable organisms recovered was similar in both groups. However, when individual organisms were considered, the mean counts for enterobacteria and veillonellae were significantly higher in the patients than in the control subjects ($P < 0.025$ and
Fig. 1. Bacteria isolated from different levels of the gastrointestinal tract in control subjects (○) and patients with tropical sprue (●). Results are expressed as the logarithm of the number of viable organisms per milliliter of intestinal juice or per gram of feces. The mean logarithmic count in each case is represented by an arrow. The numbers immediately to the right of the vertical lines indicate the number of individuals in whom the particular organism was not isolated.
P < 0.005 respectively). In control subjects, anaerobic organisms always predominated so that the ratio of aerobic to anaerobic organisms was always less than 1, whereas of the subjects with sprue, 12 had a ratio of 1 or more (P < 0.025). Of those sprue patients with a history of less than 1-year duration only 3 out of 20 had a ratio of 1 or more, whereas of those with a history of a year or more, 9 out of 14 had a ratio of 1 or more, a difference significant at the 1% level. The reason for this increased ratio is chiefly due to an increase in the total number of aerobic organisms, particularly enterobacteria, rather than to any decrease in the number of anaerobes.

Attempts were made in the patient group to correlate various parameters with individual and total flora present at the different levels of the small intestine. There was no correlation between the bacteriological findings and the duration of history, the degree of malabsorption of fat, xylose or vitamin B₁₂, the urinary indican excretion, the serum and red cell folate concentration, or the serum vitamin B₁₂ concentration. There was also no correlation between the histological appearance of the biopsy and the bacteriology. However, when villous architecture was considered, significantly fewer subjects with leaf-like villi had enterobacteria (P < 0.005) and streptococci (P < 0.025) in the upper jejunum than did those with a convoluted pattern. There was no similar correlation when other organisms or other levels of the intestine were considered. There was also a correlation between the X-ray grading and the presence of enterobacteria in the upper jejunum—significantly more patients with abnormal barium meal findings having these organisms in the upper jejunum than those with normal X-ray findings (P < 0.005). However, no such correlation was present when other organisms or other levels were considered.

All the 35 patients showed symptomatic improvement during their stay in the hospital, although the changes in absorption tests, radiological, and biopsy findings varied. Ten subjects left the hospital 3 to 6 weeks after the start of the study and could not be followed further. The remaining 23 have been followed for periods ranging from 2 to 15 months and the results of treatment are summarized in table 2.

No relationship could be detected between the course of the illness and the bacteriological findings. Patient W.G., the American with tropical sprue who had quite a severe clinical illness, had very few organisms in the jejunum and upper ileum (fig. 2) yet he apparently responded to tetracycline (fig. 3). Within 2 days after starting tetracycline therapy he experienced marked subjective improvement. His vitamin B₁₂ absorption returned to normal within 5 days, but relapsed slightly later. After 2 weeks his steatorrhea was greatly reduced but his xylose absorption remained abnormal. He subsequently suffered a mild relapse even while continuing tetracycline therapy but 11 months later was apparently cured.

Another patient with severe illness (R. M.) had significant numbers of organisms throughout the small intestine (fig. 4). With no specific therapy other than hospital diet and medication to con-

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total</th>
<th>Cured</th>
<th>Improved</th>
<th>No change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo only</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Placebo and folic acid</td>
<td>16</td>
<td>4</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Placebo, folic acid, and antibiotics</td>
<td>9</td>
<td>5</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table 2. Results of treatment in 33 patients with sprue followed for 2 to 15 months**

**Fig. 2. Patient W.G.** The numbers of most prevalent bacteria per milliliter of intestinal juice at different levels of the gastrointestinal tract on the 5th day of admission. S, stomach; J, upper and lower jejunum; I, upper and lower ileum; F, feces.
isolate bacteroides. These organisms have, however, been found by others in saliva\textsuperscript{31} and in the gingival crevice.\textsuperscript{32} Half the controls in this study had bacteria in numbers up to $10^6$ per ml in the fasting gastric juice. Similar findings were reported in normal subjects by Franklin and Skoryna.\textsuperscript{34}

In subjects living in temperate regions the upper small intestine is usually sterile or has less than $10^2$ organisms per ml,\textsuperscript{35} although occasional subjects may have $10^3$ to $10^6$ organisms per ml.\textsuperscript{36, 37}

In the lower ileum, however, mean counts

trol the diarrhea, his general condition, his absorptive function, X-ray, and biopsy findings improved remarkably (fig. 9), yet the bacteriological findings remained essentially unchanged. At the time of the third intubation his xylose absorption had decreased, he had gained some more weight, and the organisms were reduced in numbers. When seen again 7 months later he had suffered a mild relapse, yet no organisms were recovered from the whole of the small intestine (fig. 4).

Discussion

Control subjects. The pattern of bacteria isolated from the saliva of the control subjects resembles that found by Richardson and Jones,\textsuperscript{31} except that they did not

![Graph showing clinical course of patient W. G. with use of tetracycline.](image)

**Fig. 3.** Clinical course of patient W. G. Tetracycline was given (1 g per day) during the period indicated at the top of the chart. Biopsy (arrows): 1, mild changes; 2, partial villous atrophy; 3, severe partial villous atrophy.\textsuperscript{31}

![Graph comparing bacteria and coccid in a patient.](image)

**Fig. 4.** Patient R. M. Bacteria of the gastrointestinal tract depicted as in figure 2. - - - - , first intubation on day 106; -- , second intubation on day 225; - - - - - - , third intubation on day 406; - - - - , fourth intubation on day 606.

![Graph showing clinical course of patient R. M. Numbers indicating biopsy findings as in figure 3.](image)

**Fig. 5.** Clinical course of patient R. M. Numbers along the bottom indicate the days when intubation was performed.
of $10^4$ or $10^5$ organisms per ml are frequently encountered. Studies of the microbial flora of the small intestine of normal individuals living in the tropics are very limited. Nadel and Gardner sampled the upper jejunum in 13 control subjects and found bacteria in 5 (streptococci and/or micrococci and Gram-positive bacilli). Gorbach et al. studied 13 Bengali control subjects and found no coliforms in the jejunum but in 11 out of 13 these were present in the ileum with counts ranging from $10^4$ to $10^7$ per ml. In an earlier study by the same workers, out of eight controls, bac- teroides, clostridia, and anaerobic lactobacilli were present in the mid or lower ileum in 2, 6, and 1 subjects respectively.

While meaningful comparisons between results in different laboratories are difficult because of differences in sampling and culturing techniques and differences in the populations studied, it is apparent that the control subjects in this study have more bacteria, and more f other type organisms in the upper gastrointestinal tract than has been found in previous studies. The explanation for the presence of this increased flora in apparently healthy individuals with normal gastrointestinal motility is obscure. It has been shown that bacteria in the environment and in food can colonize the gastrointestinal tract. Perhaps the lack of sanitation and resultant general contamination of the environment (as exemplified by the frequently observed gross bacterial contamination of drinking water sources) may play a part in producing this observed increase in gastrointestinal flora. Gorbach et al. found that subjects with malnutrition had increased numbers of organisms, particularly coliforms, in the upper intestine and suggest that "some control patients reported by other investigators were similar to our malnourished group, and this may explain their abnormal small bowel flora." Unfortunately these authors gave no specific details regarding dietary intake, but, in that their controls were stated to have been eating a "good diet," it seems possible that they may have been eating a diet rather different from that of the controls in this study, who, although they had no clinical evidence of malnutrition, could certainly not be regarded as eating a good diet.

It is possible that the presence of these bacteria in the upper small intestine may in some way be related to the as yet unknown factor(s) responsible for the minor morphological abnormalities of the jejunal mucosa seen in these subjects and which are prevalent in many tropical regions. Alternatively it is possible that the organisms themselves are responsible for histological abnormalities such as have been shown to occur in gnotobiotic animals when bacteria are introduced into the intestinal lumen. However, in man the bacteria are more numerous lower down the intestine where morphological abnormalities are usually less marked. It therefore seems improbable that the two are directly connected. Moreover, in this small group of control subjects there was no relation between the jejunal histology and the bacteriological findings. Five of these control subjects had an abnormal xylene absorption, but this does not seem to have been due to the bacteria in the jejunum since there was no correlation between the results of this test and the bacteriological findings.

The results obtained from the stools of the control subjects agree in general with the findings of other workers, anaerobes being predominant.

Patients with sprue. The studies on the patients with sprue show that many have more than $10^4$ organisms per ml at all levels of the small intestine. However, when compared with the control subjects there are no significant differences between the two groups, either in terms of the number of individuals with or without a particular organism at a given level, or in terms of the number of organisms found. The only situation where a significant difference existed was in the stools—the patients with sprue having significantly more enterobacteria and veilonelae than the controls. The reason for this is unknown, but it presumably reflects some alteration in the luminal environment favoring the growth of these particular organisms. A
similar increase in coliforms has been noted in a study of patients with regional enteritis and severe ulcerative colitis. The explanation for the observed correlation between the presence of enterobacteria and total streptococci in the upper jejunum and the villous architecture is unknown. The organisms may in some way be responsible for the morphological changes; the presence of a convoluted mucosa may encourage the proliferation of the organisms, or both may be related to some other factor. The finding of an association between the presence of enterobacteria and radiological abnormalities is also of interest. Perhaps the altered peristalsis favors the proliferation of these organisms. However, it should be noted that enterobacteria were also present in some control subjects with no radiological abnormalities. The significance of this finding is therefore difficult to interpret.

There was no over-all correlation between the bacteriological findings, the absorption tests, and the results of therapy. Patient R. M. clearly demonstrates that, in an individual, it was not possible to relate the absorption tests to the bacterial flora or vice versa. The apparent response of W. G. to tetracycline, in spite of the presence of only minimal intraluminal flora, raises the question of the relationship of tetracycline administration to the response. Since many patients undergo spontaneous remission the condition might have pursued a similar course even without antimicrobial therapy. It may also be that the tetracycline had some function other than that mediated by its antimicrobial effect. It is also possible that sampling the luminal contents may not necessarily sample the bacteria adherent to the intestinal mucosa. The only available study in man showed no evidence of a microbial population adherent to the mucosa as distinct from the luminal one. Nevertheless it is possible that in W. G. there were such organisms which were tetracycline sensitive. Finally the tetracycline may have acted on an as yet unrecognized infectious agent which may either be the cause of tropical sprue or a secondary perpetuating factor.

Most of the previous studies of intestinal bacteria in tropical sprue have been carried out only on jejunal aspirates and with limited bacteriological techniques. Milanes et al. cultured jejunal aspirates from 19 patients and found no organisms in 7, coliforms in 7, and other organisms in 5. No controls were studied. Nadel and Gardner sampled the jejunum in normal controls and in patients with sprue and found no difference between the two groups, about 60% in each group being sterile. Klipstein et al. obtained coliforms in the jejunal aspirate in 3 out of 6 patients and Desai et al. grew organisms in small numbers in 12 of 28 Indian patients, but neither studied control subjects. Lahri et al. studied jejunal aspirates from 29 Indian control subjects and 18 patients by limited bacteriological methods and observed no significant difference between the two groups. O’Brien and England, in Caucasian subjects with sprue, found that jejunal aspirates from 7 were either sterile or showed insignificant growth. Ileal aspirates from 5 showed counts within normal limits. These findings are similar to that in the American subject in this study, but unlike the majority of the Indian subjects. Gotch et al. carried out careful bacteriological studies of Indian patients with tropical sprue. Full details of their bacteriological findings are not given but it appears that their results in sprue patients are similar to those reported here. However, their control subjects are different in that none of them had coliforms in the proximal small intestine. These authors also studied a group of 7 patients, designated as “protein calorie malnutrition” with normal fat and vitamin B<sub>12</sub> absorption, 4 of whom had coliforms in the stomach or jejunum. They conclude that there is “a spectrum of bacteriological findings” ranging from the healthy controls —with no coliforms in the upper bowel, through the malnourished group, 60% of whom had coliforms in the upper bowel, to the patients with sprue, 93% of whom had coliforms in the stomach or jejunum. Unfortunately, there are no specific details given regarding the dietary intake of any of the groups, but in that the controls were stated to have been eating a good diet it seems possible that they may have
been eating a diet rather different from that of the patients with tropical sprue and may therefore not have been strictly comparable.

From the studies reported here it is concluded that, although bacteria are frequently present in numbers of 10^9 per ml or more throughout the small intestine of patients with sprue, this cannot be considered a specific abnormality. It is possible that in some cases these luminal bacteria may contribute to the pathogenesis of the malabsorption of tropical sprue but, if so, the way in which they do it is not clear.

In the stagnant loop syndrome, the intestinal luminal bacteria may produce bile salt deconjugation which reduces the concentration of bile salts below the critical micellar concentration and thus produces steatorrhea. However, in 20 of the patients reported in this study, qualitative bile salt studies showed evidence of deconjugation in only 1 subject and that only in the lower ileum. The bacteria therefore clearly do not produce steatorrhea by this mechanism. Whether intraluminal bacteria can produce steatorrhea by some other mechanism such as the elaboration of toxic metabolites is not known, but if so, then the findings reported here could be explained either by assuming that the mucosal cells in the patients with tropical sprue are peculiarly sensitive to some toxic metabolite(s), or that the patients with sprue have as yet unrecognized organisms which are not present in the controls. In the stagnant loop syndrome bacteria also play a role in producing vitamin B_12 malabsorption, although the precise mechanism by which this occurs is not known. The rapid response of the vitamin B_12 absorptive defect to antibiotic administration in some patients with tropical sprue suggests that bacteria are also, in some way, responsible. However, in this study there was no correlation between vitamin B_12 absorption and the bacteriological findings at any level of the intestine, and the precise mechanism by which vitamin B_12 absorption is impaired and by which it is improved following antibiotic administration remains conjectural.

Finally, it should be emphasized that since tropical sprue may well be a syndrome and since it shows certain variations in different parts of the world, these findings can only be taken as representative of tropical sprue as seen in southern India.

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