In vitro cultivation of parasitic protozoa that cause human disease is invaluable, as it provides information on the development of parasites that could be used in the containment and eradication of the parasite (14). The media used for the cultivation of *Leishmania donovani* require either fetal bovine serum (FBS) (also fetal calf serum) or blood lysate as one of their essential ingredients. FBS is highly expensive (top-quality FBS can cost up to £500/liter), and reliable supply is very difficult to obtain, especially in developing countries (7). Serum can also harbor hazardous contaminants such as viruses, bacteria, prions, and mycoplasma (7). Above all, the increasing concerns about animal suffering inflicted during collection add an ethical imperative to move away from the use of serum wherever possible (7). Although several attempts have been made to replace FBS with bovine serum albumin or a mixture of purine bases, vitamins, large concentrations of certain amino acids, hormones, hemin, hemoglobin, and, more recently, even human and animal urine (1, 2, 3, 6, 9, 11, 12), none of them is widely in practice. This is due to shortcomings such as cost, complicated procedures, and laborious preparation. The collection of blood in sterile form from rabbits for Novy-MacNeal-Nicolle (NNN) medium is again a cumbersome process, and the maintenance of animal housing is another expensive burden. Hence, a cheap, easily available, and serum-free but good medium has been the dream of those who are involved in parasite culture. Ultrafilter fractions of milk have long been shown to have growth factors similar to those of serum and have been used as a substitute for serum in the media for the cultivation of hybridoma (8) and mammalian (13) cells, but again, the product reflects many of the shortcomings of FBS.

In the present study, we therefore investigated the use of milk from common cattle to replace FBS in the media for the primary isolation, cultivation, and maintenance of *L. donovani* parasites. This additive is quite inexpensive, and no sophisticated facilities are required for its preparation, making it an ideal preparation for those who are involved in *Leishmania* culture.

**Parasites.** The parasites used in this study (cultivation and maintenance) were isolated from the splenic/bone marrow aspirates of patients with kala-azar/visceral leishmaniasis who had been admitted to the inpatient ward at the Rajendra Memorial Research Institute of Medical Sciences, Patna, India, and maintained in medium 199 supplemented with 10% FBS (both were purchased from HiMedia Laboratories, Mumbai, India) by weekly passage. Only recent isolates (in the second passage) were selected for this study.

**Milk samples.** The milk samples were collected from cow (*Bos taurus*), buffalo (*Bubalus bubalis*), and goat (*Capra hircus*) in separate sterile containers, brought to the lab, and processed fresh or stored at 5 to 8°C. The milk samples, if stored, were brought to room temperature (RT) and were then exposed to boiling water for 30 min. The caps of the glass tubes were kept loose to facilitate the release of steam. After the milk samples were brought down to RT (26 to 30°C), they were kept at 3 to 4°C. The next day, the milk samples were taken out from the refrigerator and kept at RT for 2 h before being exposed to boiling water. This was done on three successive days, with an incubation of 24 h between each exposure to boiling water, a fractional sterilization method called tyndalization. Bacterial endospores that survive the initial heating will germinate in the milk while it is kept at room temperature. The resulting vegetative bacteria will be killed when the milk is reheated (4). It was noticed that, after every heat exposure, the color of the milk slowly turned to yellow. The tyndalized milk samples were centrifuged at 3,000 rpm for 30 min to remove fat globules, as they may disturb the microscopic examination. Without dis-
turbing the upper creamy layer and bottom deposits, the centrifuged milk samples were collected in separate glass tubes by a Pasteur pipette and stored in the refrigerator at 3 to 4°C. A set of tyndalized milk samples was monitored for the appearance of contamination every week by microscopic examination and by streaking in nutrient agar plates for up to 6 months. The milk samples were collected from three animals from each group for this triplicate study.

Culture methods. The cells of *Leishmania* promastigotes were sedimented by centrifugation at 4,000 rpm for 30 min and washed twice with 1.0 ml of sterile phosphate-buffered salt solution to remove any traces of FBS. The parasites were counted in an improved Neubauer counting chamber (hemocytometer) with a 400/1000 objective of light microscopy, and the number was adjusted to 1 × 10^6 parasites/ml. Medium 199 (pH 7.2; with 25 mg/liter gentamicin) was filter sterilized through a 0.22-μm filter membrane (Nalgene) by pressure passage. One hundred microliters of the inoculum containing 10^6 parasites/ml was inoculated in each of 15 tubes, 3 with 1 ml of medium 199 alone, 3 with 1 ml of medium 199 plus 10% FBS (heat inactivated at 56°C for 30 min), and 9 with 1 ml of medium 199 plus 10% milk of cow, buffalo, or goat (3 of each). Each tube of the triplicate was inoculated with one of the isolates of three different patients. The tubes were incubated at 25°C for up to 12 days. The growth of promastigotes was monitored every day through microscopy with a hemocytometer.

Milk supplement in primary isolation. Either bone marrow or splenic aspirates were collected from each of 26 patients who were suspected to be suffering from visceral leishmaniasis. The aspirated material was expelled into a 12- by 75-mm sterile culture tube containing 1 ml of sterile Locke’s solution. After mixing well, we inoculated equal amounts of aliquots in both modified NNN medium with 10% defibrinated pooled rabbit blood in blood agar base (HiMedia), with Locke’s solution as overlay, and medium 199 with the tyndalized milk (10%) of cow, buffalo, or goat (alternatively for each sample). The remaining aspirated material in the syringe was expressed onto glass slides, spread with a needle, stained with Giemsa, and examined under a microscope for Leishman-Donovan bodies. The parasite load was graded from 0 to 6, and the slides were considered negative if no parasites were detected in 1,000 microscopic fields (15). Gentamicin (25 μg/ml) was added to the media to avert bacterial contamination. The inoculated tubes were incubated at 25°C and examined on every third day for the presence of motile promastigotes by ordinary light microscopy with a 10× objective. The tubes showing no growth of promastigotes were discarded as negative after 15 days of incubation.

Maintenance of culture by weekly subpassaging. One milliliter of the number-adjusted (1 × 10^6/ml in phosphate-buffered saline) promastigote culture was transferred to the tubes with 10 ml of medium 199 plus 10% milk of cow, buffalo, or goat. Subpassaging was done on every seventh day by transferring approximately 1 ml of culture to the fresh medium. The growth of promastigotes was monitored for up to 25 subpassages.

Biochemical investigation of the tyndalized and centrifuged milk samples and FBS was carried out for the selected constituents, such as protein, glucose, triglyceroids, calcium, and cholesterol. This was done in triplicate, and the mean was obtained from the readings.

As shown in Fig. 1, the maximum growth of promastigotes of *Leishmania donovani* was achieved in medium 199 supplemented with the milk of goat, followed by that of cow and buffalo and then the controls (one is medium 199 alone, and the other is medium 199 with 10% FBS). Though the parasites survived for up to 6 days in medium 199 without FBS, this medium failed to support the growth of *Leishmania* promastigotes and there was no further multiplication (except on the second day, when there was a slight increase of cell number), as shown in Fig. 1. In medium 199 with 10% FBS, the number of promastigotes reached up to 1.9 × 10^7/ml on the fifth day of incubation.
incubation, and in medium 199 with 10% milk of goat, cow, or buffalo, the number of promastigotes reached up to $2.6 \times 10^7$, $2.3 \times 10^7$, or $2.1 \times 10^7$/ml, respectively, on the sixth day of incubation.

In primary isolation, out of the total 26 samples studied, the medium with the milk of cow, buffalo, or goat yielded positive for promastigotes in 22 samples (84.6%), the standard microscopic examination of smears gave a positive result in 18 samples (69.2%), and the NNN medium yielded positive only in 17 samples (65.4%). Out of 22 positive samples, 4 (18.2%) were positive only in milk-supplemented media. Four samples were negative in all the three methods tested, but none of the milk-containing media gave a negative result when either NNN or microscopy gave a positive result (Table 1).

The qualitative and quantitative examinations of promastigotes for up to 25 subpassages showed that they were similar to those of the same isolates maintained in parallel in NNN medium with Locke’s solution as overlay (Fig. 2). The refrigerated tyndalized milk at 3 to 4°C did not show any contamination after up to 6 months of observation. The mean value for the biochemical investigation of tyndalized and centrifuged milk samples revealed that the goat milk had the highest level of protein (5.8 g/dl) and triglyceroids (485.8 mg/dl). The buffalo milk had the highest level of calcium (42.4 mg/dl) and cholesterol (70.7 mg/dl). Only the glucose level (156 mg/dl) was found to be exceptionally higher in FBS than in the tested milk samples. The results of our study prescribe a relatively simple medium supplement which is universally available, with no barrier of prohibitive cost, no complicated procedures in preparation, and no risk of infection, and can be used in place of FBS in the in vitro cultivation of *Leishmania* promastigotes and perhaps other hemoflagellates. The medium supplemented with milk showed a remarkable increase in cell density compared to the medium supplemented with FBS and also showed a superior sensitivity in the primary isolation (diagnosis) of promastigotes. The fractional sterilization (tyndalization) of milk is easy to perform and does not require any expensive equipment. This is particularly important under field conditions and in rural areas where the facilities are scanty. As fractional sterilization is enough to remove all the contaminants, there is no chance for the presence of infectious agents. There is both moral and legal imperative (European Directive 86/609/EEC) for scientists to use alternatives to FBS whenever possible, but there is no bar to use animal-derived materials such as milk (7).

The use of milk-supplemented medium in the primary isolation of parasites from clinical materials would reduce the use of NNN medium that requires rabbit blood to be taken by painful and sometimes lethal cardiac puncture. The goat milk appears to be the most effective in transmitting amastigotes from clinical materials to promastigotes, followed by buffalo and cow milk. In milk-supplemented medium, the percentage of positivity is equal to or greater than that for standard methods. As the quantity of splenic aspirates was very small, innoc-

![FIG. 2. Maintenance of *Leishmania donovani* promastigotes in media supplemented with FBS and milk of cow, buffalo, and goat. C, control.](image)

**TABLE 1. Determination of *Leishmania donovani* in splenic and bone marrow aspirates by culture and microscopy**

<table>
<thead>
<tr>
<th>Result</th>
<th>No. of samples with result/no. of samples tested (%)</th>
<th>Medium 199 with 10% milk</th>
<th>NNN medium</th>
<th>Microscopic examination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C  B  G  Total</td>
<td>C  B  G  Total</td>
<td>C  B  G  Total</td>
</tr>
<tr>
<td>Positive</td>
<td></td>
<td>8/9 (88.9) 6/9 (66.7) 8/8 (100) 22/26 (84.6)</td>
<td>8/9 (88.9) 5/9 (55.6) 4/8 (50) 17/26 (65.4)</td>
<td>8/9 (88.9) 5/9 (55.6) 5/8 (62.5) 18/26 (69.2)</td>
</tr>
<tr>
<td>Negative</td>
<td></td>
<td>1/9 (11.1) 3 (33.3) 0</td>
<td>4/26 (15.4) 1 (11.1) 4/9 (44.4) 4/8 (50) 9/26 (34.6)</td>
<td>1/9 (11.1) 4/9 (44.4) 3/8 (37.5) 8/26 (30.8)</td>
</tr>
</tbody>
</table>

* C, cow; B, buffalo; G, goat.
ulation of the material in three tubes (for cow, buffalo, and goat) with milk-supplemented medium and NNN medium reduces the parasitic load and may affect the positivity of the sample. Hence, the clinical material was inoculated in medium with any one of the milk supplements and NNN medium.

The milk supplement could simplify the diagnosis of leishmaniasis, particularly in the rural setup. The maintenance of promastigotes is also easy and cheaper for a relatively longer period of time than with other media. As there was no contamination in the tyndalized milk stored at 3 to 4°C during the observed period of 6 months, the supplement could be prepared once and stored in the refrigerator for up to 6 months. Biochemical analysis showed that the levels of triglyceroids and protein appear to be the key factors, as both of them were comparatively higher in goat milk. Glucose seems to play a minor role in the growth of promastigotes, as its level was lowest in goat milk and highest in FBS. Further works with other Kinetoplastida need to be done which could provide a wider utility of this report, simplify the diagnostic and culture work, and reduce animal sufferings. There would be some minor differences due to “lot-to-lot” variability; a simple prior standardization needs to be done with the locally available milk samples. The only possible disadvantage of using milk as a supplement in the culture medium seems to be the light haziness of the medium. Even after the removal of the creamy layer (fat) by centrifugation, the supplement may still contain smaller protein globules, which may disturb the microscopic examination of the parasite.

We are thankful to B. K. Tyagi, the officer in charge, and R. Rajendran (CRME, Madurai, India). The technical assistance of N. Verma, M. M. Ramachandran, and V. R. Mannar is greatly acknowledged. We dedicate this work to S. K. Bhattacharya, Additional Director General, ICMR, New Delhi, India.

REFERENCES
5. Reference deleted.
10. Reference deleted.