# *Bacillus* sp. causing abscessation in sheep and goat population

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**India is a rich repository of sheep genetic resources and its diversity. Here, sheep is the major source of meat and wool; goats are reared for meat and milk. Disease out-breaks cause large economic losses each year due to the high rates of mortality and morbidity in infected sheep and goats. In the present work, abscess in the sheep and goat population was studied. The size of the abscess increased gradually up to 6.5 ± 1.5 cm in diameter; it became slight pinkish-red colour and then broke out. Also, the disease was specific to females. Gram staining performed with a thin section of the tissues of the abscess showed that the pathogen of the disease was a rod-shaped, Gram-positive bacteria. The bacterium from the abscess was isolated and the isolate was stored in the laboratory, named as 062011 msu. A series of biochemical experiments and the 16s rRNA sequence confirmed that the bacteria belonged to *Bacillus* sp. *In vitro* experiments with the pathogen showed that it was sensitive to tetracycline and ciprofloxacin. Administration of antibiotics in the infected animal for a week dissolved the abscess completely.**

**Keywords:** Abscess, *Bacillus* sp., 16s rRNA gene, sheep pathogen.

INDIA has the third largest sheep population in the world[1], with about 40 breeds of sheep. They are affected by many diseases such as foot rot disease, foot scald disease, external parasite diseases, internal parasite diseases, mastitis, clostridial diseases and listerosis caused by worms, bacteria, fungi, viruses and other external factors[2]. Abscess formation has been reported in sheep and goat population. *Mycobacterium* and the *Corynebacterium pseudotuberculosis* form external abscessation. Caseous lymphadenitis is a chronic lymph node infection in both sheep and goats, which is caused by *C. pseudotuberculosis*. Abscessation of both internal and external lymph nodes is possible in the diseases[3].

In the present work, abscess in the sheep and goat population was studied. Statistical analysis of the disease shows that it is female-specific. Gram staining performed with a thin section of the tissues of the abscess showed that the pathogen of the disease was a rod-shaped, Gram-positive bacteria. The bacterium from the abscess was isolated and the isolate stored in the laboratory under the name 062011 msu. The bacteria was found to belong to the *Bacillus* sp. *In vitro* experiments with the pathogen showed that it was sensitive to tetracycline and ciprofloxacin. The administration of antibiotics for a week dissolved the abscess completely.

A sheep flock from Maruthamputhur village near Alangulam Region, Tirunelveli District, Tamil Nadu (lat. 8.83546° and long. 77.51509°) was used for the study and the infected sheep were carefully maintained. Seven types of sheep were reared in sheep farm and the flock had 200 sheep. All the sheep were fed with grass from a nearby forest and agriculture land; water was available in the farm and in the nearby natural ponds.

All experimental procedures were approved by the ethical committee of Manonmaniam Sundaranar University, Tirunelveli, India. The abscess was collected from the infected sheep, with the help of a sterile surgical blade.

The Gram-staining method was performed using the HIMEDIA Gram-staining kit. Briefly, the culture smear was prepared on a clean glass slide. Then, a drop of crystal violet was added and it was incubated for 2 min at room temperature. The excess stain was washed with tap water. The sample was incubated with Gram's iodine for 2 min. The slide was rinsed with water and decolourized with 95% ethanol. Finally, the sample was covered with a few drops of safranin for 2 min. After brief washing with tap water, the slide was air-dried. Then the slide was observed under the microscope.

The bacterial culture was smeared, heat-fixed and flooded with carbol fusion stain. The slide was heated for 3–5 min and cooled followed by washing with distilled water. The smear was decolourized with acid–alcohol for 10–30 s until the slide becomes faint pink; it was then washed with water. Finally, the sample was subjected to counter stain by covering it with methylene blue solution for 1–2 min. The excess stain was washed with distilled water and the slide was dried using a blotting paper. It was then observed under the microscope.

The collected abscess portion of the tissue was fixed in 10% formalin fixative for 24 h. The fixed tissue was washed with water and then the sample was subjected to the dehydration process, which was accomplished by passing the tissue through a gradient of isopropyl alcohol from 70% to 100%, followed by clearing the isopropyl alcohol using xylene and embedded with paraffin wax (HIMEDIA). Then, a thin section of 7 μm thickness was obtained using a microtome (Besto). The section was processed and subjected to haematoxylin–eosin basic staining protocol. The slide was mounted with DPX (distrene plasticizer xylene) and observed under the microscope for documentation.

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In order to identify the biochemical characteristic of the pathogen, the following biochemical tests were performed using the HIMEDIA kit: spore formation test, methyl red, catalase, urea hydrolysis, citrate, oxidase, starch hydrolysis and casein hydrolysis test, according to the manufacturer's protocol.

For molecular characterization of the organism, genomic DNA was isolated from the bacterial isolate using phenol–chloroform extraction method[4]. The quality of the genomic DNA was checked using UV spectrophotometer. In order to amplify the 16s rRNA gene, universal primers (forward primer: GGTTACCTTGTTACGACTT and reverse primer: AGAGTTTGATCCTGGCTCAG) were used and PCR were performed[5]. Then the amplified PCR product was purified using the DNA purification kit (GeneJET™ Gel Extraction Kit, Cat. No: #K0691, Fermentas, USA). The amplified DNA was sequenced.

The 16s rRNA sequence of the pathogen and other bacteria was aligned using Align X software and the phylogenetic tree was constructed[6].

Among the 200 individuals of sheep and goat, there were six different breeds of sheep. 1. Ganjam, 40 in number; female – 28; male – 12 (34 adults and 6 young ones); 2. Madras Red, 40 in number; female – 23; male – 17 (27 adults and 13 young ones); 3. Kenguri, 30 in number; female – 19; male – 11 (24 adults and 6 young ones); 4. Kilakarsal, 35 in number; female – 22; male – 13 (27 adults and 8 young ones); 5. Meechari, 20 in number; female – 14; male – 6 (15 adults and 5 young ones); 6. Vembur, 25 in number; female – 18; male – 7 (18 adults and 7 young ones). The goat variety was called as Kodi adu, 10 in number; female – 6; male – 4 (7 adults and 3 young ones; Figure 1 *a*). The abscess was found in Kilakarsal (35%; 12/35) and in Kodi adu (10%; 1/10), which was the least infected breed among the sheep and goat population (Figure 1 *b*). It was also found that all the infected animals were adult females, and the abscess was not observed in any male and young ones. Generally, the abscess is found all over the body in an infected individual, except the head, ear and udder. Interestingly, a single abscess was found on the hind leg of an infected sheep (Figure 2 *a*). The mean size of the abscess was 6.5 ± 1.5 cm in diameter. A close-up view of the abscess is shown in Figure 2 *b*. The gradual increase in the size of the abscess was noted. Finally it became slightly pinkish-red in colour. Then, the abscess was broken and the pus was released along with the fluid as shown in the Figure 2 *c*. The abscess had caused reduction in milk production and also loss of body weight in the sheep. The infected animals which were not administrated with suitable antibiotics died. The tissue of an early abscess of an infected individual was subjected to thin sectioning by histopathological technique as described earlier. The section was stained with eosin and hematoxylin. Figure 3 *a* illustrates the presence of enlarged nucleus and loss of cells in the infected tissue, whereas in the control tissue the nucleus remains normal. A thin section of the abscess was subjected to the Gram staining. Figure 3 *b* clearly shows the presence of rod-shaped bacteria inside. The

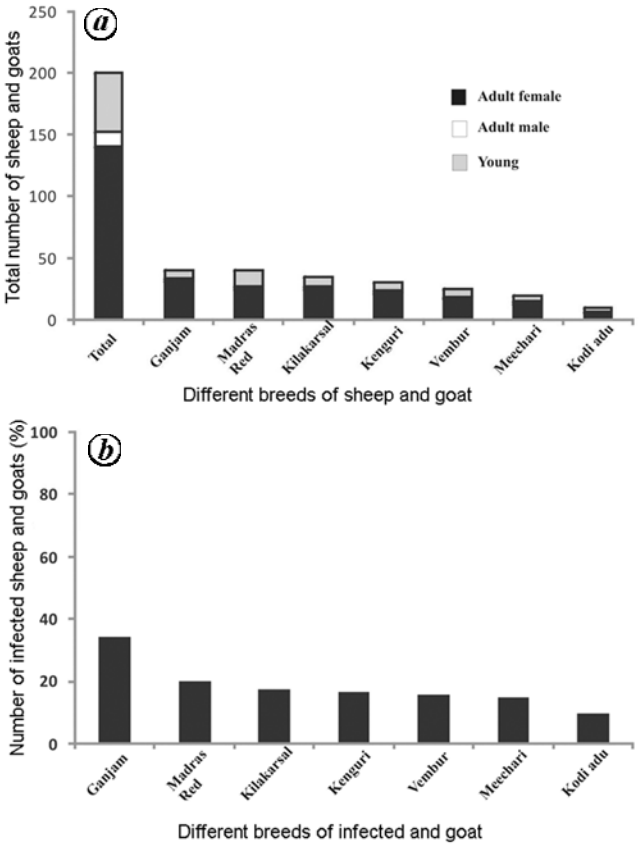


**Figure 1.** ***a***, The sheep and goat population in the herd. The young refers to goat or sheep which are below four months of age. ***b***, The infected sheep and young goat in the herd. The Kilakarsal was the most susceptible, while Kodi adu was the least. All the infected animals were adult females.

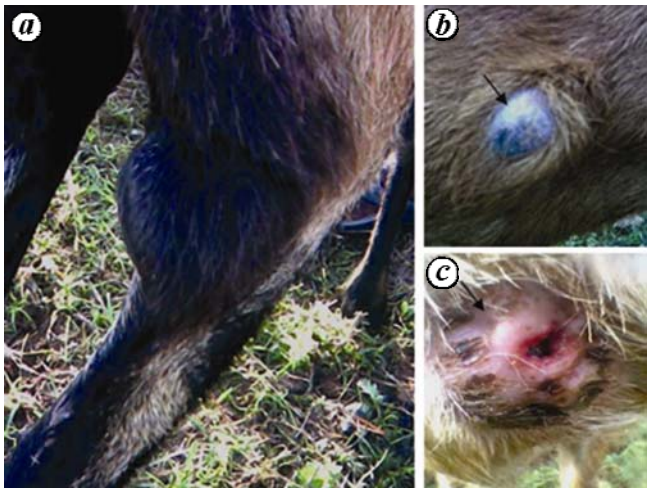


**Figure 2.** The abscess in the sheep and goat population. ***a***, The abscess in the right thy with the mean size of 6.5 ± 1.5 cm in diameter. ***b***, The arrow mark shows the clock up view of the abscess. ***c***, The burst out abscess denoted by arrow.

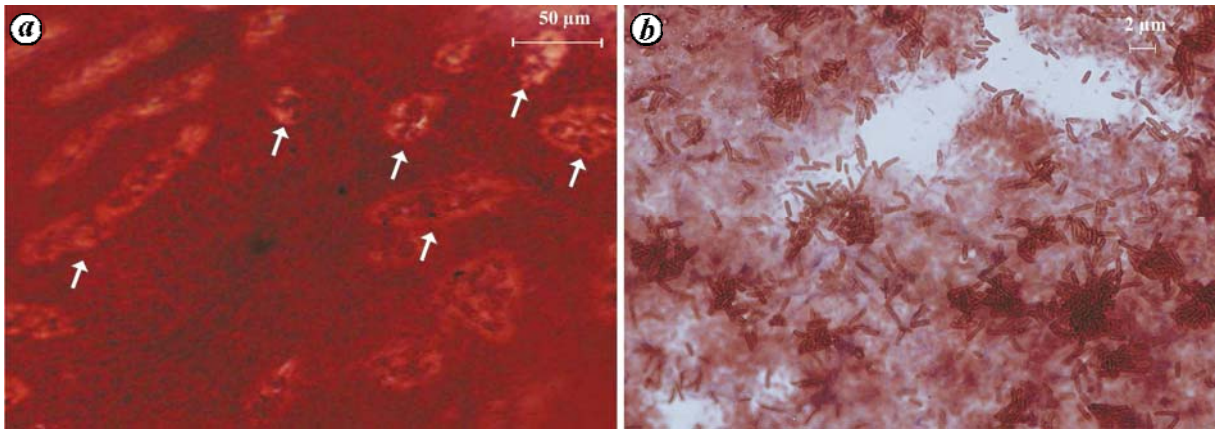


**Figure 3.** Histopathological studies of the control and infected tissue samples. ***a***, Double staining of the infected tissue. The enlarged nuclear structure is shown by arrows and the loss of cells in the infected tissue is shown with arrowhead. ***b***, Gram staining of the infected tissue.

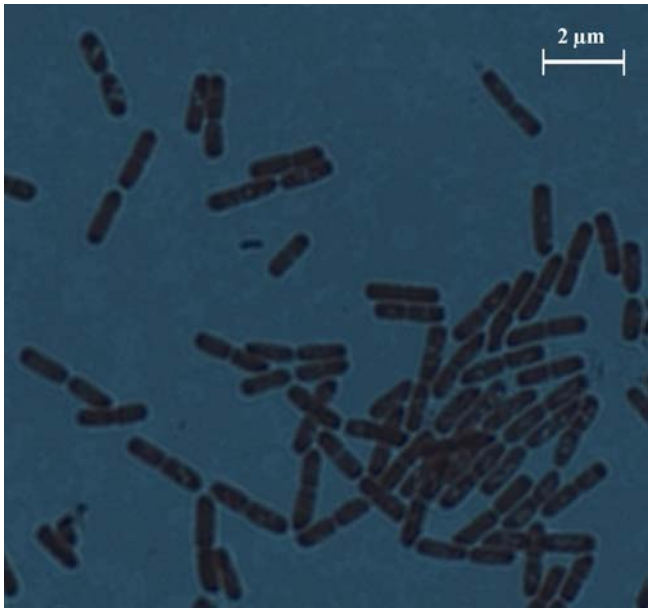


**Figure 4.** Gram-stained pure culture of the pathogen. The pathogen was identified as Gram-positive, rod-shaped bacteria.

**Table 1.** Summary of the biochemical tests

| Test | Observation |
|---|---|
| Indole | – |
| Methyl red | + |
| Voges–Proskauer | – |
| Catalase | + |
| Urea hydrolysis | + |
| Citrate | + |
| Starch hydrolysis | – |
| Casein hydrolysis | – |
| Oxidase | – |
| Gram staining | + |
| Acid fast staining | – |
| Spore formation | + |

data confirm the causative agent of the abscess as Gram-positive bacteria. The bacterium was 2 ± 0.5 µm in length and 0.5 ± 0.2 µm width.

The hair around an early abscess was removed by shaving with a razor blade. Then, the area was surface-sterilized using 70% ethanol, and about 2 cm deep biopsy was taken. The biopsy was sterilized by washing in sterile PBS using the serial dilution technique. It was homogenized under aseptic condition in sterile nutrient broth and filtered through a cheese cloth. Then the bulk debris of the tissues was removed by differential centrifugation technique. The supernatant was spun at 5000 rpm for 10 min. The obtained pellet was suspended in sterile nutrient and plated in nutrient agar plate. Two colonies were obtained, which were subjected to Gram staining (Figure 4). The data clearly show that the two colonies were Gram-positive, rod-shaped bacteria. The shape and size of the bacteria of the pure culture (Figure 4) and of those seen in the histological section of the infected abscess (Figure 3) were morphologically the same. The two colonies were subjected to a series of biochemical tests. Table 1 provides a summary of the tests and the results are shown in the 'supplementary data' (Supplementary figures 1–9; available at online). From the data, it can be inferred that the two colonies obtained are the same. In addition, the pathogen was subjected to spore formation test as described earlier. The pathogen did not form any spore for a week. The subterminal endospore was noted on the 8th day of incubation of the slant inoculated with the pathogen at 37°C (Figure 5 *a*). On the 10th day, the vegetative cells were not seen, and the complete spore was observed (Figure 5 *b*). Following the *Bergey's Manual of Bacteriology*[8], the isolates were identified as *Bacillus* sp.

*C. pseudotuberculosis* is a well-known abscess-forming pathogen for the sheep and goat population. Hence to further confirm the species of the isolated organism, the genomic DNA of the pathogen was isolated using the standard protocol as described earlier. The PCR reaction was performed using the universal primer for 16s rRNA gene. The PCR product was purified and sequenced with the forward primer. Then, the sequence was

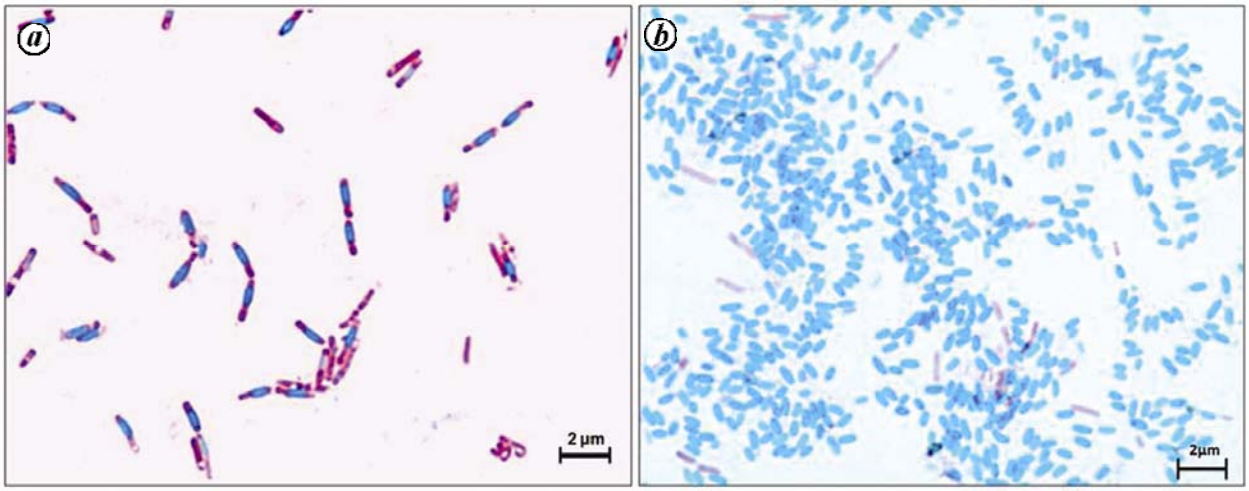


**Figure 5.** Spore formation test. ***a***, The pathogen was observed to form a subcentral endospore on the 8th day after inoculation. ***b***, On the 10th day after inoculation, a few vegetative cells were observed. The spore and vegetative cells were stained with blue and pink colour respectively.

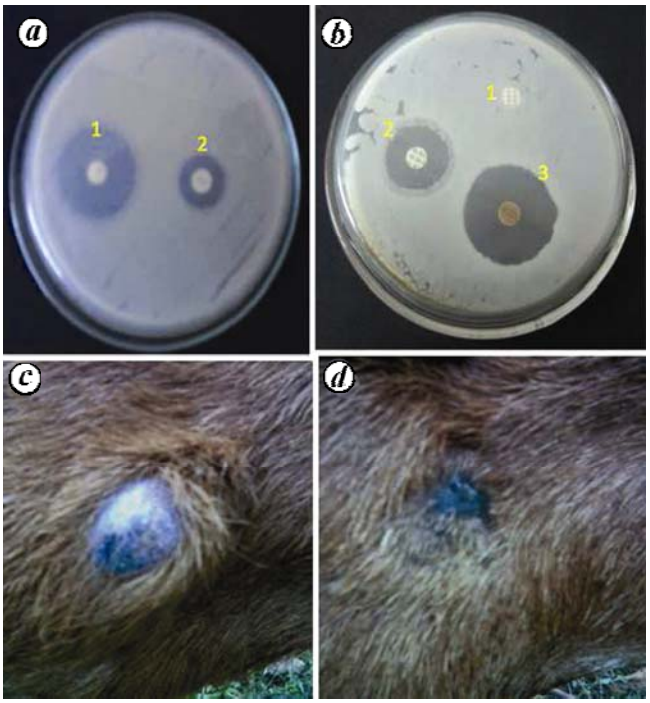


**Figure 6.** Sensitivity of the pathogen to antibiotics. ***a***, ***b***, *In vitro* experiments: ***a***, (1) Ciprofloxacin and (2) Gentamicin; ***b***, (1) Ampicillin, (2) Amikacin and (3) Tetracycline. ***c***, ***d***, *In vivo* experiments: ***c***, The abscess before antibiotic administration. ***d***, Dissolution of the abscess on the 7th day of antibiotic administration.

aligned with the already reported sequence of 16s rRNA gene of 25 different bacterial species (Supplementary figure 10). The molecular biological data (Supplementary figure 10 *a*) and the phylogenetic tree (Supplementary figure 10 *b*) confirm that the pathogen belongs to the *Bacillus* sp. Further analysis of the 16s rRNA sequence of the pathogen with different *Bacillus* species (Supplementary figure 11) confirms that the pathogen is genetically similar to *Bacillus anthracis*. This is an obligate, Gram-positive, central endospore-forming, rod-shaped bacterium, 1–1.2 µm in width and 3–5 µm in length and is the causative agent of anthrax[7]. The spore of *B. anthracis* is highly resistant to high temperature and low-nutrient environments, and it survives for several years in the harsh environmental conditions[8]. Because the 16s rRNA sequence of the pathogen did not match with any species of *Bacillus* (Supplementary figure 11), it has been concluded that the organism is a novel species of *Bacillus* sp. and named as *Bacillus* sp. 062011 msu.

Then the pure culture of the pathogen was subjected to antibiotic sensitivity assay. A total of seven antibiotics were screened for the *in vitro* experiment, as shown in Figure 6 *a* and *b*. The pathogen *Bacillus* sp. was resistant to the following antibiotics: ampicillin, amikacin, trimethoprim, and gentamicin. The pathogen was sensitive to tetracycline with 10 mm diameter zone of inhibition (Table 2). The second best among the tested antibiotics was ciprofloxacin with 9 mm zone of inhibition. As a final step, to test the antibiotics which have the potential to inhibit the pathogen *in vivo*, 10 different infected individuals were administrated with 100 mg/20 kg tetracycline twice a day for 7 days orally. During the course of the antibiotics, a reduction in the size of the abscess was observed (Figure 6 *c* and *d*). Finally, the abscess was completely dissolved in a week's time. Similarly, 100 mg/20 kg ciprofloxacin was administrated twice a day for 7 days for 10 different sheep and the abscess completely disappeared in a week's time. The data clearly confirm that tetracycline and ciprofloxacin are the potent antibiotics to cure the disease caused by the pathogen, *Bacillus* sp. Thus the finding of *Bacillus* sp. adds one more genus other than *Corynebacterium* which can form abscess in the sheep and goat population.

**Table 2.** Sensitivity of the pathogen for different antibiotics

| Antibiotic | Sensitivity | Zone of inhibition (mm) |
|---|---|---|
| Tetracycline | Sensitive | 10 |
| Ciprofloxacin | Sensitive | 9 |
| Chloramphenicol | Sensitive | 7 |
| Amikacin | Sensitive | 4 |
| Gentamicin | Sensitive | 3 |
| Trimethoprim | Resistant | – |
| Ampicillin | Resistant | – |

Sheep and goat farming is a source of income for a large population of small and marginal farmers in India. Sheep have the most important economic value in the world because of their use as food, wool and leather. The observation of abscess in the goat and sheep population suggests that only the adult female is the victim of the disease. Usually infants are susceptible to bacterial diseases. For example, pertussis most commonly affects infants or young children and can be fatal, especially in babies less than 1 year of age[9]. Chlamydia is a bacterial disease affecting sheep, goats and cattle. The new born sheep is preferentially infected by chlamydia[10]. Streptococcal septicemia is a severe bacterial infection that affects newborn infants[11]. Watery mouth disease develops quickly and predominantly kills new born lambs[12]. Tuberculosis is a chronic bacterial disease in animals and humans characterized by the progressive development of specific granulomatous lesions of tubercles in the affected tissues. The disease affects all age groups of susceptible hosts and is accountable for more deaths throughout the world than any other bacterial disease ever today[13–18]. However, the abscess caused by the newly characterized *Bacillus* sp. was not observed in any infant or male sheep and goat in the farm. The reason for the sex specificity is not yet known. However, this knowledge is important to control the disease in the farm.

For the penicillin-based antibiotics, the isolates of *Bacillus* sp. did not respond efficiently when compared with that of the antibiotics tetracycline and ciprofloxacin. For the treatment of *Bacillus* sp. infected sheep and goat, tetracycline or ciprofloxacin at the concentration of 100 mg/20 kg injected twice a day for 7 days was enough to completely cure the abscess-forming disease. It has been reported that the treatment of *C. pseudotuberculosis* is more difficult and there is a lack of complete efficient treatment for the infected sheep and goat. However, for *Bacillus* sp. infected sheep and goat, the remedy is simple, efficient and cost-effective to cure the disease completely.

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