Coincidence of Haemorrhagic Septicemia and Rabies

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SUMMARY
An unusual sickness in a cattle confusing with symptoms of haemorrhagic septicemia (HS) was reported to the institute in the periphery of Kasoor District. Animal was showing typical symptoms of HS but was also very furious and biting the other animals. A team of researchers from the institute visited the place and collected the samples and information in all aspects related to any disease occurrence on epidemiological basis.

Blood and saliva samples were collected for laboratory testing. Biochemical tests including indol production, glucose and sucrose fermentation tests regarding HS confirmation and any blood born disease were done. Saliva sample was tested for ruling out rabies. Reverse transcriptase polymerase chain reaction (RT-PCR) by amplifying “N” region gene and mouse inoculation test (MIT) were performed and it was very astonished that animal was positive for both HS and rabies.

Key words: Haemorrhagic septicemia; Rabies; RTPCR; MIT.

1. INTRODUCTION

Haemorrhagic septicaemia (HS) is an acute, highly fatal septicemic disease of cattle and buffaloes (characterized by dullness, depression, reluctance to move, elevated temperature, salivation, nasal discharge and edematous swellings in the pharyngeal region and then spread to the ventral cervical region and brisket (1, 2, 3, 4, 5). Visible mucous membranes are congested and respiratory distress is soon followed by collapse and death (6). If animal is suffering from immunosupression due to any protozoal disease or some bacterial or viral disease, then it will be favorable for the attack of Pasteurella multocida (7, 8, 9, 10, 11, 12). The annual loss due to HS disease in Asia was estimated, one laccs animals (2). Haemorrhagic septicaemia is caused by specific serotypes within the bacterial species of Pasteurella multocida (9, 19).

Pasteurella species are spherical, ovoid or rod-shaped, Gram-negative and occur singly, or in pairs or short chains. Bipolar staining may be seen and capsules may also be present. All species are non-motive and are facultative anaerobic (7, 8) worked with 58 strains from buffalo and bovine in India, Pakistan, Thailand and Kenya belonging to type -I. (3) used indirect haemagglutination test and recognized four serotypes and designated them as A, B, C and D.

Rabies is a highly fatal viral infection of the central nervous system, which occurs in all warm-blooded animals including man. In Greek, the word rabies means, “madness”. Rabies virus is the prototype species of the genus Lyssavirus in the family Rhabdoviridae. Virion is a single stranded, negative sense, non-segmented RNA (15). The physical appearance of this rigid, rod like, often cone-shaped or bullet-shaped structure of the rabies virion, with one end flattened (planer) and the other rounded (hemispherical) and surrounded by lipid-protein envelope (5, 13, 14).

All species of livestock are susceptible to rabies, cattle and horses are the main frequently affected animals (1). Rabies virus is highly neurotropic in the infected host causing a fatal encephalomyelitis. Few days before the onset of symptoms, the virus is present in large quantities in the saliva of the affected animal. Rabies is transmitted only when the virus is introduced into bite wounds, open cuts in skin or outer mucus membranes form saliva or other potentially infectious material such as neural tissue (4). Severe and multiple bites to the head and neck and bites to highly innervated areas may result in shorter progresses through a short prodromal stage to encephalopathy and an ascending paralysis leading to the death of the victim usually within days (16). With the advent of newer molecular techniques particularly PCR and its modifications, one expected that it would increase the specificity and sensitivity of antemortem diagnosis of rabies. The development of reverse transcriptase polymerase chain reaction (RT-PCR) methodology has facilitated greatly the genetic characterization of many rabies viruses (RVs). The relative temporally conserved nature of certain regions of the RV genome; particularly N gene permits development of rapid molecular methods for RV typing. In recent years, RNA detection by RT-PCR has been suggested as a rapid and sensitive method for rabies diagnosis (17).

An unusual sickness in a cattle confusing with symptoms of HS was reported to the institute in the periphery of Kasoor District. Animal was showing typical symptoms of HS but was also very furious and biting the other animals.
The most common symptoms observed in cattle were: difficult breathing, abnormal vocalization, biting the other fellows and drooling of saliva. A team of researchers from the institute visited the place and collected the samples (blood, faecal and saliva) for diagnosis of the disease. Two days after collection of samples, animal was found dead. Post mortem was conducted and different organs were collected along with femur bone to get information, in all aspects related to any disease occurrence.

2. MATERIALS AND METHODS

(i) Isolation and identification

Samples were collected aseptically and transported to the institute immediately. Blood smears were stained with Giemsa stain to rule out any blood born parasite. As the animal was suspected for H5 and Rabies, the samples were processed for the confirmed diagnosis of these diseases, as well.

Trachea, lungs and femur bone marrow were selected for the bacterial isolation. Trachea and lungs were inoculated on blood agar and MacConkey agar and incubated at 37°C. Trachea, lungs, along with bone marrow were triturated in normal saline and inoculated into mice. Mice were found dead 24-48 hrs post inoculation and impression smears from dead mice hearts’ were stained by gram staining method to determine the morphological patterns of the bacteria.

Different biochemical tests like indol production, glucose and sucrose fermentation tests, as described by (10) were conducted to further confirm the pathogen.

Saliva sample was subjected to MIT and RTPCR for the purpose of ruling out the occurrence of rabies.

Test procedures for rabies diagnosis.

1. Mouse inoculation test (MIT)

Mouse inoculation test was performed as described by (11). Briefly, a 10% brain tissue suspension by weight was prepared in phosphate buffered saline (PBS) solution. Centrifuged the tissue suspension for 5 minutes at 150-200g to remove the gross particles. Fifteen to twenty days old Swiss albino mice were procured from mice colony of the institute. A volume of 0.03ml of brain tissue suspension to remove the gross particles. Fifteen to twenty days old Swiss albino mice were procured from mice colony of the institute. A volume of 0.03ml of brain tissue suspension (10%) was inoculated intracerebrally to individual mouse. Five mice were inoculated with suspected material and one mouse was kept as a control in each set. Mice were checked daily for 21 days. Deaths occurring 24-28 hours after intracerebral inoculation were attributed to the causes other than rabies virus, such as: trauma, bacterial contamination or other viruses.

2. Reverse transcriptase polymerase chain reaction (RT-PCR)

- Reverse transcriptase polymerase chain reaction was performed, as described by (18) using the most highly conserved region of “N” protein gene (443 bp). Primer sequences used were the following:
  - N1 (+) sense: (587) 5’- TTT GAG ACT GCT CCT TTT G-3’ (605)
  - N2 (-) sense: (1029) 5’- CCC ATA TAG CAT CCT AC-3’ (1013)
  - N1 primer was used to prime cDNA, which was thereafter amplified by the N1-N2 set. 

Isolation of RNA: Each saliva sample was washed with distilled water, added 1mL of TRIzol-reagent to 100 mg of sample and kept for 5 minutes after grinding the material. It was put on ice for 5 minutes, added 200 µL chloroform into the same eppendorf tube and put on ice for 15 minutes. Thereafter it was centrifuged at 40 C for 15 minutes at 12,000 rpm, took liquid phase and added to it 600 µL of isopropanol and mixed it inverting and put on ice for 5 minutes. Afterwards, it was centrifuged at 40 C for 10 minutes at 12,000 rpm, discarded the isopropanol and pellet was observed. RNA pellet was washed with 75% ethanol and centrifuged at 40 C at 9,000 rpm for 5 minutes, air dried and dissolved in a nuclease free 100 µL DEPC treated water, kept at 550 C for 10 minutes in a water bath (e.g.using chemicals/enzymes/kits of Fermentas ®).

cDNA preparation;

Step-I:
- RNA 5 µL
- Reverse primer 1 µL
- DEPC treated water 2 µL
- Incubated at 650°C for 5 minutes in thermocycler (Advanced primus 96 peqlab®).
- Chilled on ice.

Step II:
- 5X RT buffer 4 µL
- dNTPs 8 µL
- Incubated at 370 C for 5 minutes in thermocycler.

Step III:
- Added reverse transcriptase 1 µL
- Incubated at 420°C for 60-90 minutes.
- PCR of cDNA;
- Following mixture was prepared;
  - cDNA 5 µL
  - dNTPs 4 µL
  - DEPC treated water 30 µL
  - Reverse primer 1 µL
  - Forward primer 1 µL
  - Taq Buffer 5 µL
  - MgCl2 3 µL
  - Taq polymerase 1 µL

This mixture of 50 µL was programmed to following PCR conditions: five initial cycles of denaturation (60 seconds at 94°C), annealing (90 seconds at 450C, then 20 seconds at 50°C) and elongation (90 seconds at 72°C) and 30 additional cycles, where denaturation and elongation were reduced to 30 seconds and 60 seconds, respectively. Final elongation was carried out at 72°C for 10 minutes. Final product was subjected to gel electrophoresis using 1% agar gel and results were viewed using gel documentation system of BioRad® (Model: Universal Hood II).

3. RESULTS

P. multocida organisim was isolated from the samples of the animal suspected for haemorrhagic septicemia. After primary isolation, the isolates were confirmed by Gram’s staining, different biochemical tests, mice inoculation and re-isolation. Gram-ve, oval & rod shaped bacteria were observed under microscope. Some bacteria were found in chains, while others were found as a single entity. The characteristics, which confirmed the pathogen as P. multocida included: no growth on MacConkey agar, no hemolysis.
on blood agar, indol production, and glucose and sucrose fermentation without gas production. Examination of the blood smears of the infected animal revealed that the animal was suffering from trypanosomiasis. Saliva sample was processed for MIT and RTPCR. Sample was found positive for rabies by these tests. Positive mice for rabies showed ruffled fur, tremors, lack of coordination of hind legs, ascending paralysis, prostration and finally death.

Specific bands of 443 bp were observed by amplification of “N” region gene through RTPCR, as shown in a figure. Saliva samples collected from healthy controls, as well as the normal rabbit brain samples were negative, indicating the specificity of the primers used.

4. DISCUSSION

As the animal was showing the typical symptoms of HS, but was also very furious and biting other animals confusing with rabies and latter on found dead. The death of the animal may be due to suffocation caused by P. multocida. The blood examination of the animal showed that it was suffering from trypanosomiasis. Animals infected with trypanosomiasis suffer from the immunosuppression as observed by (12). Immunosuppression lead to the favorable conditions for the attack of P. multocida and its reproduction in the animal.

Trypanosomiasis in the animal might have caused the immunosuppression, which provided favorable conditions for the development of P. multocida, which might have caused the suffocation. The incubation period of rabies virus, ranges form few days to months (i.e. depending upon the site of bite and viral load). In this case the animal might be attacked by any rabid animal in this period and animal remained unattended till the terminal stage of the disease. Suffocation, due to P. multocida, as well as severe shock due to rabies and trypanosomiasis might be the cause of the death of the animal.

5. CONCLUSION

Biochemical tests, including: indol production, glucose and sucrose fermentation tests, regarding HS confirmation and any blood born disease were performed. Saliva sample was tested for ruling out rabies. Reverse transcriptase polymerase chain reaction (RT-PCR) by amplifying “N” region gene and mouse inoculation test (MIT) were performed and it was very astonished that animal was positive for both HS and rabies.

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