DIAGNOSIS OF HAEMORRHAGIC SEPTICAEMIA AND AVIAN PASTEURELLOSIS USING POLYMERASE CHAIN REACTION

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Haemorrhagic septicaemia (HS) is an acute septicaemic disease principally affecting cattle and buffaloes. Disease characterized by a rapid course, edematous swelling in the head, throat and brisket region, swollen and haemorrhagic lymph nodes and presence of numerous sub serous petechial hemorrhages. Two specific serotypes of Pasteurella multocida are responsible for this highly fatal acute septicaemic disease. In Asia HS is caused by serotype B: 2 and in Africa by serotype E: 2. In India B: 2 is the most prevalent serotype.

On the basis of distribution of the disease, three distinct categories of countries have been identified. India comes under the category 'A' where the disease is endemic and is of utmost economic importance (OIE Animal Health, 2002).

Resource allocation for prevention and control of HS reflects the correct estimate of its economic impact. Epidemics of HS may occur as alarming and devastating disease problems in cattle and buffalo and jeopardize not only the economic return of production animals, but also affect draught power of animals. In Kerala, there are reports on outbreaks of HS from almost all the districts of the state, even though the total number of animals affected and deaths due to HS are very less and the increased incidence of the disease is mainly found in the rainy seasons.

Pasteurella multocida affects and causes great economic loses in a wide spectrum of hosts, including poultry and domestic ruminants like sheep and goats, besides cattle and buffaloes. In poultry, there are reports of outbreaks due to Pasteurella multocida among ducks (Duck pasteurellosis) and in fowls (Fowl cholera). Pasteurella multocida can cause septicaemic disease in domestic birds like turkeys and quails also, besides ducks and fowl.

Avian pasteurellosis is caused by serotype 'A' Pasteurella multocida. In sheep and goats cases of pneumonia due to P. multocida had been reported.

Ducks, especially the indigenous varieties, are generally considered to be more hardy than chicken. However, disease outbreaks do appear in them from time to time. A study conducted by the department of Microbiology, COVAS, Mannuthy as part of the ICAR scheme on "Development of Cell Culture Duck Plague vaccine for simultaneous vaccination with duck pasteurellosis", has revealed that outbreaks were either due to simultaneous infection with duck plague virus (DPV) and Pasteurella or Pasteurella alone. Most of the outbreaks among foraging ducks have been attributed to pasteurellosis.

Fowl cholera (FC) has also been reported in the state, although the incidence is far less compared to duck pasteurellosis. Effective commercial vaccines are available for FC, but there is no information regarding the use of these vaccines in ducks. Hence, there is no systematic vaccination for pasteurellosis in ducks in the state and the disease continues to be a major threat to duck farming.

There are reports about the significant association of P. multocida in the nasopharynx of apparently normal bovines and outbreaks of HS. Hence detection of P. multocida in apparently healthy domestic ruminants (carrier status) can be considered as an indicator of the susceptibility of the herd for HS.

To treat a particular disease and to adopt effective preventive measures against the same, the causative agent has to be found out as quickly as possible. Conventional methods for detection of P. multocida causing avian pasteurellosis and HS rely on the detection of the **bipolar organism** by **microscopy** and its **isolation and identification**. These methods although confirmatory, are time consuming, often taking at least a week and are labour intensive.

The limitations faced in the isolation and identification of bacteria can be overcome by employing techniques like nucleic acid based assays, which serve as alternate methods of bacterial identification.

Nucleic acid based assays allow detection of organisms, both dead and live, in clinical samples with utmost sensitivity and specificity, thereby decreasing the time required for bacterial identification. Polymerase Chain Reaction (PCR) has been practically useful in this regard, with the use of primer sequences designed to facilitate identification at any level of specificity, strain and species.

POLYMERASE CHAIN REACTION FOR DETECTION OF P. multocida

The essential requirements for carrying out PCR, are two synthetic oligo nucleotide primers (forward and reverse, ~ 20 nucleotides each), a target sequence in a DNA sample that lies between the pair of primers which can be from 100-5000 bp in length (template DNA), A thermo stable DNA polymerase (Taq DNA polymerase) and the four-deoxy ribonucleotides (dNTPs) - dATP, dGTP, dCTP and dTTP.

Each PCR cycle will have three basic steps 1) Denaturation-

thermal denaturation of the DNA sample by raising the temperature within a reaction tube to 94°C 2) Renaturation (Annealing) - the temperature of the mixture is slowly cooled to 55°C and the primer base pair anneal with their complementary sequence in the source DNA 3) Synthesis (Extension) - temperature of the mixture is slowly raised to 72 °C, which is the optimum temperature for the catalytic functioning of the Taq DNA polymerase. DNA synthesis is initiated at the 3' OH of each primer and each single strand gets converted to a double stranded DNA molecule.

Pasteurella multocida species specific (PM–Specific) PCR can be used for detection or confirmation of P. multocida in suspected clinical samples from cases of HS or avian pasteurellosis. **Serotype B specific** (HS-B Specific) PCR can be used for confirmation of serotype of the isolate especially from cases of HS. **Multiplex PCR** with the help of which confirmation of species and serotype of P. multocida in a single step using PM-specific primers and HS-B specific primers simultaneously in the same reaction also can be carried out.

Primers for detection of bacterial/viral pathogens can be selected from already published articles or can be self designed. More than a dozen software packages are available in the internet for this purpose. PRIMER 3 developed by S.E Lincoln et.al is available free over the net.

For the synthesis of primers, sequence can be given to commercial companies. In India, **Bangalore GENEI**, **Alpha DNA (Canadian) through Medox Agencies**, and **Finzymes through Labmate** are some of the examples of the firms that can be relied.

For example, for detection of P. multocida by PM–Specific PCR, forward primer KMT1SP6 (Sequence 5 '- GCT GTA AAC GAA CTC GCC AC- 3') and the reverse primer KMT1T7 (5'-ATC CGC TAT TTA CCC AGT GG - 3) is used and for detection of P. multocida by Type B Specific PCR, forward primer KTSP61 (5 '- ATC CGC TAA CAC ACT CTG - 3') and the reverse primer KTT72 (5 '- AGG CTC GTT TGG ATT ATG AAG – 3'.) can be used. These primers are selected from already published work of Townsend et. al (1998). For multiplex PCR, two sets of primers each (those for PM-PCR and HS-B PCR) can be used simultaneously in the same reaction.

Template DNA

PCR can be performed using template DNA prepared from a) P. multocida cultures obtained after isolation, b) morbid materials such as heart, liver, and spleen collected from poultry during post mortem, c) blood samples and blood smears collected at height of temperature from cattle and buffaloes suspected with HS and goat suspected with pasteurellosis, d) blood smears and impression smears collected at post mortem from poultry e) nasal swabs collected from apparently healthy ruminants (to study carrier status) and clinically ill animals showing respiratory symptoms and f) lung samples showing pneumonic lesions from slaughtered ruminants.

Blood samples (one to two drops of blood from jugular vein) from suspected cases of HS can be collected into sterile vials containing 0.5 to 1 ml of defibrinated ovine/bovine blood and nasal swabs can be collected using sterile cotton swabs into 2 ml of sterile phosphate buffered saline (PBS).

Colony touch method

The PCR assay can also be performed on suspected bacterial colonies after isolation trials; single bacterial colony can be picked up with the help of a sterile pipette tip and added directly to the PCR master mix.

Method of preparation of template DNA

From P. multocida cultures

Inoculate a pure colony of P. multocida into five millilitres of brain heart infusion broth (BHIB) and incubate at 37°C for 18 h. One point five millilitres of this broth culture can be transferred to an Eppendorf tube and centrifuge at 3000 X g in a cooling centrifuge for 10 min. Wash the pellet twice in PBS (pH 7.4) and resuspend the final pellet in 100 µl of triple distilled water. Boil the mixture for 10 min and immediately chill on ice for 30 min. Thaw the sample and centrifuge at 3000 X g for 5 min. The supernatant fluid can be stored at -20°C for further use as template DNA.

From morbid materials

Two grams of tissue samples (heart, liver, spleen) can be homogenized in two millilitres of PBS in sterile mortar and pestle. Keep the mixture undisturbed in a refrigerator for 20 min to settle large tissue debris. Boil the supernatant for 10 min and immediately chill on ice for 30 min. After that centrifuge at 3000 X g for 10 min. The supernatant fluid can be stored as above.

From blood smears and impression smears

Blood smears and impression smears collected at post mortem can be scrapped with a blade into an Eppendorf tube. To this tube add 1.5 ml of sterile distilled water and keep the mixture at 37°C for 30 min. Centrifuge at 3000 X g for 15 min. Wash the pellet twice with PBS and resuspend the final pellet in 50µl of triple distilled water, boil for 10 min and immediately chill on ice for 30 min, thaw and centrifuge at 3000 X g for 10 min. Store the supernatant at -20°C.

From blood samples

Take two hundred microlitres of blood into an Eppendorf tube. Add sterile triple distilled water to make up the volume to 1.5 ml. Rest of the procedure same as that of blood smears except that final pellet after washing with PBS was resuspended in 100 µl of triple distilled water.

From nasal swabs and lung samples

Inoculate nasal swab and representative portion of lung samples into 3 ml of BHIB and incubate at 37°C for three hours. Mix the tubes containing inoculated broth for 3 minutes in a vortex mixer. After that 1.5 ml of this broth culture can be transferred to an Eppendorf tube and rest of procedure same as that employed

for preparation of template DNA from P. multocida cultures.

SETTING UP OF PCR (TEST PROPER)

Polymerase chain reaction can be performed in a total volume of 25 µl reaction mixture. Preparation of master mix can be done according to the number of samples to be tested. For testing two samples, master mix must be prepared for four reactions, one for negative control with distilled water, one with known positive control and two for clinical samples to be tested.

Preparation of 20 µl master mix for four reactions can be carried out by mixing the following reagents in the given proportions; PCR reaction buffer (10x)-10 µl, Forward primer and reverse primer 4 µl each, dNTP mix- 8 µl, Taq polymerase- 2µl and triple distilled water to make 80 µl.

All these reagents can be procured from firms like **Bangalore Genei, Medox agencies, Vision Scientific** etc, supplying molecular biology chemicals.

This master mix will be divided into **four** 0.2 ml thin walled PCR tubes, 20 µl into each tube. To first tube add 5 µl of distilled water (negative control), to 2nd and 3rd tube 5 µl of template DNA prepared from suspected clinical samples, to 4th tube template DNA prepared from known isolate or reference strain of P. multocida (known positive control for comparison)

The PCR amplification can be carried out in an automated thermal cycler. The programme followed for PM-PCR is, initial denaturation at 95°C for 4 min, followed by 30 cycles of denaturation at 95°C for 45 sec, annealing at 55°C for 45 sec and extension at 72°C for 45 sec and a final extension at 72°C for 6 min. The whole reaction will be conducted under the heated lid.

For HS-B PCR, initial denaturation at 95°C for 4 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min and a final extension at 72°C for 9 min.

For multiplex PCR, the same programme for HS-B PCR can be followed.

When the programme is over in PCR machine, the contents in PCR tube is called as amplified product, which can be transferred to a freezer -20 °C or -70 °C before analyses or to a fridge (4 °C) if analyses is done on the same day. If kept at -20 °C or -70 °C the amplified product will remain intact for several months.

DETECTION OF PCR PRODUCTS

The amplified products of PM- PCR, HS-B PCR, Mutiplex PCR can be detected by submarine agarose gel electrophoresis. Agarose gel (1.5 per cent) prepared in Tris borate EDTA (TBE) buffer can be used. Molecular markers like pBR 322/ Alu Idigest can be used to assess the size of migrated fragments of DNA. Electrophoresis will be carried out using electrophoresis apparatus at 5V/cm for one hour. The gel can be visualized under UV transilluminator and results will be documented in a gel documentation system.

ANALYSES OF RESULTS OF PCR

Presence of Pasteurella multocida DNA in suspected clinical samples can be confirmed by the amplification of 460 bp fragment in PM-PCR (Fig.1). In the negative control, no amplification product will be detected.

If the clinical sample is obtained from suspected cases of HS, HS-B PCR can be performed directly or after doing PM-PCR. Since HS is caused by serotype-B P. multocida an amplified product of approximately 590 bp fragment in HS-B PCR will give a confirmatory diagnosis (Fig. 2). Avian pasteurellosis is caused by serotype A P. multocida; no amplified product will be detected for clinical samples from suspected cases of avian pasteurellosis in HS-B PCR.

If multiplex PCR is done using template DNA prepared from suspected cases of HS and avian pasteurellosis simultaneously, using both the sets of primers (PM-PCR and HS-B PCR) in same reaction, two bands will be obtained for serotype B P. multocida at 460 and 590 bp positions while serotype A will give only one band at 460 bp position (Fig. 3).

CONCLUSION

Polymerase Chain Reaction as a molecular biology technique has allowed bacterial detection directly from clinical specimens, dramatically reducing the time required for identification. It can be used to obtain a rapid and confirmatory diagnosis of P. multocida, without the need to obtain pure cultures and conduct biochemical tests. ❑

LIVESTOCK ASIA: 2007 VENUE CHANGED TO THE NEW KUALA LUMPUR CONVENTION CENTRE

Asia's International Feed, Livestock and Meat industry show- Livestock Asia 2007 Expo & Forum will be moved to the new prestigious Kuala Lumpur Convention Center from its original venue at MIECC, Mines Resort City. This is a biennial event hosted by the Department of Veterinary Services, Ministry of Agriculture and Agro –based Industry Malaysia and supported by the Veterinary Association Malaysia and Malaysian Feed Millers' Association. More than 250 exhibitors world wide including major integrators such as CP, Leong Hup, Ayam Wira will once again make their presence at this show. Complementing the expo is the 4 th Feed and Livestock Industry Conference which has been recognized as the top industry gathering in the region.