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DIAGNOSIS OF HAEMORRHAGIC SEPTICAEMIA: PAST, PRESENT AND FUTURE

T.K. Dutta, Rajeev Gautam, V.S. Senthil Kumar and S.K. Kotwal*

Division of Veterinary Microbiology & Immunology
*Division of Veterinary Public Health and Hygiene
SKUAST-J, R. S. Pura, Jammu – 181 102

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ABSTRACT

Haemorrhagic septicaemia (HS) is a distinct bacterial disease of cattle and buffaloes, and is of economic importance in some parts of world including India. The causal agent of the deadly disease is Pasteurella multocida (serotype B:2 in Asia and E:2 in Africa). Accurate laboratory diagnosis and subsequent characterization (typing) of the causative organism by traditional methods are time consuming, laborious, costly and sometime provide ambiguous results. The development of DNA-based techniques has provided an alternative methods of detection and characterization that overcome the limitations of traditional methods. Two polymerases chain reaction (PCR) tests have been reported for detection of P. multocida. Both tests show promising and encouraging outcome as diagnostic tool. Till date, there have been various techniques used for typing of P. multocida isolates which include: restriction endonuclease analysis (REA), ribotyping, pulse field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD) assay, repetitive extragenic palindromic–PCR (REP–PCR), multilocus enzyme electrophoresis (MLEE), amplified fragment length polymorphism (AFLP) and multi-locus sequence typing (MLST). All the new generation techniques are having certain limitations and constraints. These techniques are not suitable at all for isolation and are best used in parallel with other conventional techniques. At least two distinctly different methods should be used to give any inference.

Keywords: Diagnosis, haemorrhagic septicaemia, review

INTRODUCTION

Haemorrhagic septicaemia (HS) is an acute disease infecting cattle and buffalo (water & swamp) caused by two specific serotypes of *Pasteurella multocida*. The Asian serotype is designated B:2, and the African serotype is E:2 by Carter-Heddelston system, corresponding to 6:B and 6:E by Namioka-Carter system. The disease is characterized by a rapid course of oedematous swelling in the throat and

brisket region, swollen and haemorrhagic lymph nodes and the presence of numerous subserous haemorrhages. HS is considered economically to be the most important bacterial disease in South-East Asia including Indonesia, Philippines, Thailand, Malaysia, Middle-East, North-East, Central and South Africa [1]. In India, it is prevalent in all states of the country, and the high risk areas in general are parts of Rajasthan, Gujarat, Karnataka, Andhra Pradesh and Assam [2]. The disease ranks at number one amongst bacterial diseases with huge annual mortality in cattle and buffaloes, besides in other domestic animals [2, 3].

Accurate laboratory diagnosis of *P. multocida* depends on the isolation and identification of suspected bacterial colonies by microscopy and biochemical tests. Samples taken immediately from animals after death yield almost pure cultures of *P. multocida* from e.g. heart blood, spleen, liver, bone marrow or lung. However, isolation of *P. multocida* can prove difficult during field surveys of carrier status when the samples are taken from contaminated sites, such as the nose or throat. Extensive subculturing is essential to obtain a pure culture of the causative organism and a long time is required for the preparation of antisera to conduct the current *P. multocida* serotyping. This is not a practical approach for HS prone endemic countries like India. This often leads to a prolonged lag phase between the collection of materials and serotype identification [4]. These problems could be circumvented by applying the sensitive and specific molecular biological techniques especially polymerase chain reaction (PCR) [5]. For characterization of *P. multocida*, the other molecular biological techniques such as Restriction Endonuclease Analysis (REA), Ribotyping, Field Alternation Gel Electrophoresis (FAGE), Randomly Amplified Polymorphic DNA (RAPD)-PCR, Repetitive Extragenic Palindromic (REP)-PCR, Plasmid profile analysis and other new emerging techniques are being extensively used with encouraging results.

Till date, a sizeable number of excellent reviews or overviews are published on diagnosis, based on traditional cultural and typing methods etc. in different ways [6-9]. Recently Hunt *et al*. [10] also described the molecular biology of *P. multocida* in a very precise form. The present review highlights the recent developments in the techniques for easy and fruitful diagnosis and characterization of *P. multocida* causing HS.

2. THE DISEASE HAEMORRHAGIC SEPTICAEMIA

2.1 Historical Perspective

Haemorrhagic Septicaemia (HS) as a disease was first described by Bollinger

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in 1878 characterized by acute septicaemia, subserous haemorrhages with heavy mortality in stags, wild hogs and cattle [11]. The infectious nature of the disease was established by French and Friedberger in 1881 and the causative agent was isolated and named as *Bacterium multocidum* by Kitt in 1885. The term HS was first used in 1886 by Hueppe, a German pathologist and named the organism as *Bacterium septicaemia haemorrhagicae*. Trevisan in 1887 suggested the generic name *Pasteurella septica* with the animal of origin to be indicated with it where necessary. The name *P. multocida* was suggested by Rosenbusch in 1937 which is now widely accepted and has found in general usages [10]. Mutters *et al*.[12] have proposed a reclassification of the genus *Pasteurella* on the basis of DNA homology. They proposed three subspecies of *P. multocida* (*P. multocida* subsp. *multocida*, *P. multocida* subsp. *septica* and *P. multocida* subsp. *gallicida*). The causal agent of HS would under this proposal be designate as *P. multocida* subsp. *multocida*.

2.2 Economic Losses

The greatest economic losses due to HS are recorded in South-East Asia [10]. After successful eradication of Rinderpest (RP) in Asia, HS became a disease of great economic importance. The disease occurs mostly in areas where the husbandry practices are primitive. In the absence of a disease surveillance system it is often difficult to get a reliable estimate of the losses which could be very high than the reported losses. A survey carried out by Bain *et al.* [13] in Sri Lanka confirmed the existence of HS mostly in buffaloes. In Thailand, the recorded cattle deaths range from 10, 000-40,000 per annum. Outbreaks of HS have been reported by Francis *et al*.[14] and Mustafa *et al*.[15] in Zambia and Sudan, respectively. In India, the HS is the number one bacterial diseases of cattle and buffalo causing huge economic losses [2,3].

2.3 Disease characteristics, it's treatmnet and control

Classical HS is caused by *P. multocida* serotype B:2 in Asian countries and E:2 in African countries. But the other serotypes are also recorded from HS and animal pasteurellosis cases of cattle, buffalo, sheep, goat, pig poultry, deer, tiger, lion and dog, like A:1; A:3; A:3,4; A:3,4, 12; B:2; D:3; D:1; F:1 and F:3,4,12 [5, 16].

The course of the disease is generally short with average incubation period of approximately 30 hours. The typical clinical syndrome is characterized by three phases viz., the initial phase of temperature elevation with inappetance and sometimes salivation. The second phase of respiratory distress with profuse salivation and nasal discharge. And the third phase of recumbancy leading to

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terminal septicaemia. These phases are seen distinctly only when the course of the disease is long. Often under field conditions the initial phase of temperature elevation is not observed. In most of the cases, the submandibular oedema becomes evident and may spread to the brisket region and occasionally down to fore limbs. At necropsy the first obvious lesion is the subcutaneous infiltration of yellow serosanguineous fluid in the submandibular, throat, pharyngeal and brisket region. The lymph nodes are generally enlarged and when cut reveal small haemorrhages.

HS leaves very little opportunity for treatment due to its rapid onset and short courses and the treatment is only effective if carried out in the very early stages [11]. The practical approach (with difficulty) by applying sulphadimidine (33.33% solution) intravenously is common. Tetracyclines or Strepto-penicillin by intramuscular route also gives good results.

In countries where HS is endemic, vaccination is the principal means of prevention and control of the disease. Several vaccines are available: formalin killed whole cell vaccine, aluminium hydroxide gel vaccine, oil adjuvant vaccine as well as live attenuated vaccine obtained by serial passages through pigeons [1]. A live vaccine based on an antigenically related strain of *P. multocida* serotype B:3;4 isolated from a fallow deer in England 7protected calves against a challenge with serotype B:2 nine months after vaccination [16]. Verma and Jaiswal [1] have extensively reviewed the vaccines against HS.

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3. DIAGNOSIS OF HAEMORRHAGIC SEPTICAEMIA

The laboratory diagnosis is based on the isolation of the specific causative organism from blood or bone marrow of the dead animals and its identification, and serological typing. Blood smears stained with Gram's, Leishman's or Methylene blue stains reveal Gram negative bipolar organisms. But no conclusive diagnosis, however, can be made based on direct microscopic examination of blood smear alone. The organism is usually detectable in blood cultures only in the terminal stages prior to death.

The sequence used as routine diagnostic laboratory method in Sri Lanka is as follows : animal material → mouse inoculation → mouse blood culture and examination of blood smear → rapid slide agglutination test.

3.1 Diagnosis of hamemorrhagic septicaemia by specific PCR *Assays*

Since 1985, the basic principle of *in vitro* nucleic acid amplification through

repetitive cycling showed the tremendous applications in all the fields of fundamental and applied clinical studies [17]. The PCR technique is now being routinely used for specific detection or diagnosis of infectious agents. However, for the diagnosis of HS, till now two specific PCR assays are available viz. *P. multocida* species and type specific PCR.

3.1.1. P. multocida species specific PCR assay

For development of *P. multocida* species specific PCR (PM-PCR), all the credit goes to two pioneer workers and their groups [4, 19]. The later one developed the oligonucleotide primers that amplify the *pst* gene encoding the P6 like protein of *P. multocida* which is having a significant similarity with the P6 protein of *Haemophilus influenzae* and *H. parainfluenzae*. Though the results are questionable but the technique gave encouraging results. The other more widely used technique has higher sensitivity and simplicity. This group [4] had developed the oligonucleotide primer sequence unique to clone KMT1 isolated by subtractive hybridization. The sensitivity of the PCR developed by Kasten *et al* [19] is minimum 10 organisms and additional hybridization with *pst* is required. But the technique developed by Townsend *et al* [4] can detect less than 10 organisms and there is no need of any additional hybridization for optimal sensitivity. The PCR assay applied for detection of *P. multocida* by using either genomic DNA as template or the bacterial colony or by using the field samples such as nasal swabs [20], morbid materials like spleen, bone marrow, heart blood [5]. These recent techniques have drastically reduced the time for diagnosis of the diseaes besides being more specific than the traditional system [21].

3.1.2. HS specific PCR assay

HS specific PCR has also been developed independently by Townsend *et al* [4] and Brickell *et al* [22] using different gene sequences as oligonucleotide primers. PCR technique developed by later group mainly detected the specific serogroup-B causing HS and also one of the two serogroup E *P. multocida* isolates analysed [10]. Oligonucleotide primers KTSP61-KTT72 developed by former group identified the serotypes B:2; B:2, 5 and B:5 by amplifying the fragment of ~600 bp. Minimum detection level of bacteria is similar to PM-PCR. The major advantages over the conventional isolation and serotyping is that for this technique there is no need of pure culture. Because, PCR detection method has already been established from contaminated materials, including heart blood, spleen, bone marrow etc. [5]. In India, atleast two publications are available from Indian Veterinary Research Institute, Izatnagar regarding PCR diagnosis of HS [23, 24]. In India [5] and abroad

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[20] peoples are already applying the multiplex PCR (using both the primer sets i.e. PM-PCR & HSB-PCR in the same reaction mixture) for detection of HS causing organism.

HS in cattle and buffalo is mainly caused by serotype B:2 in Asian countries, but other serotypes like, A:1; A:3; F:3, 4 are also isolated from cattle and buffalo exhibiting the symptoms of HS [16]. This condition has questioned the validity of HSB-PCR assay which can only detect the serogroup B. So, till now the PCR assay independently is not matured enough to confirm the diagnosis of HS promptly, but can detect only the presence of *P. multocida* by PM-PCR assay very accurately.

4. CHARACTERIZATION OF *P. MULTOCIDA*

Numerous studies for characterization of *P. multocida* have been taken up but with variable results. Till the development of molecular techniques, the emphasis was serotyping. Roberts in 1947 attempted to give the first classification system for *P. multocida* based on passive mouse protection test [11]. In currently available typing system, two popular methods are followed, based on the capsular and somatic antigens. There are total 5 capsular (A, B, D, E, F) and 16 (1-16) somatic antigens detected so far, and the organisms are classified by mentioning both the figures, such as for HS, it is B:2 (Carter & Heddelston system) or 6:B (Namioka & Carter system). The phenotypic characterization systems (by means of morphology, biochemical typing, serotyping etc.) are very much laborious and time consuming. Even after capsular and somatic antigen determination, few isolates react similarly for both the antigens posing problems in conclusive remarks [24]. The phenotypic typing systems provide insufficient information regarding epidemiological studies of HS. The DNA based techniques have provided the alternative methods of characterization overcoming the limitations of phenotyping [25]. In this review, an attempt is made to highlight the recent developments in the techniques for proper typing of *P. multocida* based on the current knowledge of molecular biology.

4.1. Restriction endonuclease analysis (REA)

Restriction endonuclease analysis (REA) has been successfully used as a tool for differentiation of strains in a variety of bacterial infections including those caused by *P. multocida*. Several restriction enzymes for this purpose have been described from time to time [26-31]. Restriction endonucleases cleave the DNA at specific nucleotide sequences and produce a set of DNA fragments which, upon electrophoresis separate into a characteristic banding pattern or fingerprint of the respective genome.

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To elucidate a clear picture of strain differentiation of *P. multocida*, several enzymes have been used such as H*ha* I[5, 32, 33], H*pa* II [5, 32, 34], S*ma* I [35, 36], B*gl* II [37], P*st* I [29], E*cor*R I[29]. *Hha* I and *Hpa* II have given best resolution for *P. multocida* of which *Hpa* II is better than *Hha* I [5, 33]. REA typing by *Hha* I followed by typing with *Hpa* II to further subdivide the *Hha* I REA types has been suggested as an alternative tool for serotyping [32], though the combination could not differentiate the vaccine strains CU and M9 from each other.

4.2. Ribotyping

Ribotyping in conjunction with REA has been widely used to characterize and differentiate the *P. multocida* isolates [5, 26, 29, 31, 38, 39]. This is one of the finest technique for typing based on REA. The banding pattern produced by REA are not clear, thereby making visual interpretation difficult. But REA followed by additional bybridization with a labeled DNA probe, it becomes easy to read the banding pattern and give the necessary interpretation. The probe may be labeled either by radio active or non-radioactive materials. rRNA probe is widely accepted for hybridization and subsequent interpretation [40]. Several workers have demonstrated considerable genomic heterogeneity providing sufficient evidence to discount the relatedness of outbreaks previously indistinguishable by serotyping and biotyping [10].

4.3. Field alternation gel electrophoresis (FAGE)

This technique is also known as Pulsed-Field Gel Electrophoresis (PFGE). It is a method of fingerprinting with high specificity and precision. Conventional electrophoresis, which used a constant current, cannot resolve the large fragments generated by rare cutting restrition enzymes. But in PFGE, where the electric field across the gel is changed periodically, can effectively separate the large size DNA fragments on size basis. PFGE analysis has consistently shown the greater discrimination in identification of bacterial species than ribotyping [41-43] but it has had a limited application in the typing of *P. multocida* isolates [43-46]. The major drawbacks of this technique are the requirements of highly purified intact DNA and a specialized and expensive electrophoresis equipment, which is generally not available in veterinary diagnostic laboratories doing routine work.

4.4. PCR-Bases techniques for characterization

In recent years, several works about the use of PCR based fingerprinting techniques for medical and veterinary pathogens have been reported. To mention, a few widely acceptable techniques are Randomely Amplified Polymorphic DNA

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(RAPD) –PCR, Repetitive Extragenic Palindromic (REP)-PCR and Enterobacterial Repetitive Insertion Consensus (ERIC)-PCR. REP elements are 33 to 40 base pair repeats that are present as 500 to 1000 copies accounting for upto 1% of the genome [46] and are present in a wide range of bacteria [47]. As the REP elements are distributed widely across the genome, it produces a multiple banding pattern. Several workers have reported the characterization of *P. multocida* isolates by REP-PCR [20, 48-53].

4.5. Other emerging typing methids

Recently, a number of new techniques have been developed to type the bacterial organisms. Theses include Multilocus Enzyme Electrophoresis (MLEE), Amplified Fragement Length Polymorphism (AFLP) and Multilocus Sequence Typing (MLST). MLEE is not a genotypic method but in fact a phenotypic method that examines a variation in the electrophoretic mobility of water soluble enzymes. AFLP technique combines the merits of REA and selective PCR, that amplifies some of the fragments generated during the REA stage. The technique is usually performed using fluorescent labeling and automated DNA sequencing equipment [54]. MLST uses the DNA sequencing of the gene loci to directly detect the genetic variation that results in amino acid sequence variation in the enzymes [55] and has high reproducibility and discriminatory power. Till date, there are very few or no reports regarding the characterization of *P. multocida* isolates using MLEE [55 – 57]. Moreover, AFLP and MLST appear to have a promising future.

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