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Virulence gene profiling and antibiotic resistance pattern of Indian isolates of *Pasteurella multocida* of small ruminant origin

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ABSTRACT

Pasteurellosis in small ruminants affects the livelihood of small and marginal farmers of India. The present study was undertaken to understand the trends in gene carriage and antibiotic resistance pattern of *Pasteurella multocida* isolates recovered from small ruminants over a period of 10 years in India. A total of 88 *P. multocida* isolates of small ruminant origin were subjected to virulence gene profiling for 19 genes by PCR and antibiogram study employing 17 different antibiotics. Virulence genes like e*xbB*, *exbD*, *tonB*, *oma87*, *sodA*, *sodC*, *nanB* and *plpB* (100% prevalence) and *ptfA* and *hsf-2* (>90% prevalence) were found to be uniformly distributed among isolates. Unexpectedly, a very high prevalence (95.45%) of *pfhA* gene was observed in the present study. Dermonecrotoxin gene (*toxA)* was observed in 48.9% of isolates with highest occurrence among serotype A isolates and interestingly, one of each isolate of serotype B and F were found to carry this gene. Antimicrobial susceptibility testing revealed 17.04% isolates to be multidrug resistant. Amongst all the antibiotics tested, most of the *P. multocida* isolates were found to be susceptible to enrofloxacin and chloramphenicol. This study highlights novel epidemiological information on frequency and occurrence of virulence genes among Indian isolates from small ruminants.

1. Introduction

Pasteurella multocida belonging to the family *Pasteurellaceae* is associated with a number of economically important diseases like haemorrhagic septicaemia in cattle and buffalo; enzootic bronchopneumonia in cattle, sheep and goats; atrophic rhinitis in swine; fowl cholera in poultry and snuffles in rabbits [1,2]. In addition, this bacterium has also been isolated from various cases in domestic as well as wild animals [3].

P. multocida has been classified into five types (A, B, D, E and F) based on capsular typing and most often each capsule type has been found to be predominantly associated with a specific disease in a host species [4]. However, over the years, there are reports of occurrence of uncommon capsular type from different host species (reviewed in [2]), which is either due to spontaneous change from one capsular type to another or due to cross species infections [5,6]. Such an ability of the bacteria to colonize, survive and subsequently infect multiple hosts is a great concern, which necessitates a detailed study on isolates from different host origin. In India, animal husbandry practices like cohabitation of various species of animals including poultry and use of common grazing ground may lead to spread of infection among all such host species. This results in a

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complex scenario of circulation of various bacterial strains among members of host populations, notwithstanding an increased possibility of isolates jumping species barriers.

Though antibiotics have been used traditionally for the treatment of pasteurellosis, their prolonged and indiscriminate use has led to onset of resistance among various strains [7–9], thus eroding the antibiotic armamentarium and limiting therapeutic options [10]. Most of the antibiotic resistance genes are present on bacterial plasmids, integrons and transposons, which lead to the spread of these genes among isolates [10]. Antimicrobial resistance of *Pasteurella* isolates varies according to host origin, time of infection, geographical location, anti-bacterial pretreatment and on accessibility of the isolates to the resistance genes present in the gene pool [10]. Hence, it was necessary to carry out a detailed study of the antibiotic susceptibility pattern of *P. multocida* isolates.

Virulence profiling has been used as a typing method for characterization of bacterial pathogens [11] including *P. multocida* [1,9,12]. However, studies regarding the population structure and virulence gene patterns of isolates from small ruminants are scanty [1,13,14]. Therefore, the present investigation was undertaken to study the population characteristics of *P. multocida* isolates obtained from small ruminants by determining their virulence-associated gene profiles and phenotypic antibiotic resistance pattern.

2. Materials and methods

2.1. Bacterial isolates

Eighty eight (88) *P. multocida* isolates recovered from small ruminants (sheep, $n = 67$ and goat, $n = 21$), maintained at Division of Bacteriology & Mycology, Indian Veterinary Research Institute, Izatnagar were used in this study. The isolates used in the study were categorized by host species origin, serotyping, year and place of origin. If more than five isolates were obtained from a particular farm/place, then only two representative isolates were selected to be included in the study. The location of the farms (district-wise) and the clinical status of the animal (when available) have been incorporated in the supplementary table.

2.2. Culture and biochemical tests

The isolates were revived by 18–24 h incubation in brain heart infusion (BHI) broth at 37 °C and plated subsequently onto blood agar to study cultural characteristics. They were then tested for purity (biochemical tests: indole, citrate, MR, VP and sugar fermentation tests) as per standard techniques [15].

2.3. Molecular confirmation of P. multocida by P. multocida specific PCR (PM-PCR) and capsular typing

The genomic DNA was isolated by CTAB method [16]. The isolates were reconfirmed as *P. multocida* by PM-PCR [17] followed by capsular typing using the primers employed by Townsend et al. [18]. The sequences of the primers used in the PM-PCR and capsular PCR are given in Table 1.

2.4. Detection of virulence associated genes by PCR

All the isolates were screened for carriage of 19 virulence associated genes by uniplex PCR. The PCR was performed with 1 μl of DNA, 2.5 μl of 10× PCR buffer (containing 20 mM $MgCl_2$), 0.5 μl of 25 mM $MgCl_2$, 0.5 μl of 10 mM dNTP mix, 1 μl each of 10 pM primers, 0.25 μl of 5 U Dream *Taq* DNA polymerase (Fermentas) and the final volume was made up to 25 μl using nuclease free water. PCR mixture without DNA was used as negative control. The PCR products were analyzed in 1% agarose gel along with 100 bp DNA ladder (Gene Ruler–Fermentas) by staining with ethidium bromide. The amplified product was visualized under UV light and documented by gel documentation system. The details of the virulence genes and sequences of the oligonucleotide primers are listed in Table 1.

2.5. Antibiotic susceptibility

Each of the above isolates were tested for susceptibility against 17 different antibiotics viz. ampicillin (A), amoxycillin (AM), cephalexin (CN), cefotaxime (CE), ceftriaxone (CTR), cephalothin (CH), erythromycin (E), amikacin (AK), kanamycin (K), tetracycline (T), gentamicin (G), ofloxacin (OF), pefloxacin (PF), ciprofloxacin (CF), enrofloxacin (EX), chloramphenicol (C) and co-trimoxazole [sulfamethoxazole with trimethoprim] (CO) using disc diffusion method [19]. These antimicrobials were recommended in OIE terrestrial manual chapter on haemorrhagic septicaemia and/or used by field veterinarians in different parts of India and have proven their clinical efficacy [20–22]. The test was performed by disc diffusion method recommended by Clinical and Laboratory Standards Institute (CLSI; formerly the NCCLS) and the interpretations were carried out as per CLSI standards (Performance Standard for Antimicrobial Disk and Dilution Susceptibility Test for Bacteria Isolated from Animals M31-A2) [23]. The ranges for susceptible, intermediate and resistant for each drug used in this study are given in Table 2.

2.6. Statistical analysis

Statistical analysis of the data generated from the study was performed with SPSS 16.0 (SPSS Inc., Chicago). *P* values of <0.05 were considered as statistically significant.

3. Results

3.1. Identification of the organism

The bacteria produced small, glistening, non-haemolytic, dew drop like colonies on blood agar, while no growth was observed on McConkey lactose agar plates. All the isolates were positive for catalase, oxidase, indole tests and negative for citrate, MR and VP tests. In PM-PCR, all the isolates showed amplicons of ~460 bp in agarose gel electrophoresis confirming these isolates to be *P. multocida*. Multiplex PCR of the above mentioned