

Molecular epidemiology investigations in 8 Romanian outbreaks of rabbit Pasteurellosis by pulsed-field gel electrophoresis

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BARAITAREANU STELIAN^{1,3*}, SIMONA IVANA¹, MIHAELA ZAULET², MARIA RODICA OTELEA³, ELENA ROTARU³, IPATE JUDITH⁴, DOINA DANES^{1,3}

1: Institute of Comparative Medicine, University of Agronomical Science and Veterinary Medicine Bucharest, Splaiul Independentei 105, 050097, Bucharest, Romania.

2: Faculty of Biology, University of Bucharest, Splaiul Independentei 91-95, 050097, Bucharest, Romania.

3: Faculty of Veterinary Medicine, University of Agronomical Science and Veterinary Medicine Bucharest, Splaiul Independentei 105, 050097, Bucharest, Romania.

4: Center of Study and Research for Biodiversity from Animal Genetic Resources Academy David Davidescu Romanian Academy, Calea 13 Septembrie, no. 13, 050711, Bucharest Romania

*Corresponding author: Institute of Comparative Medicine, Splaiul Independentei 105, 050097, Bucharest, Romania, Tel.: +4021-4011188, Fax: +4021-4011188, e-mail: doruvet@yahoo.com

Abstract

*Pasteurellosis is one of the most serious diseases of rabbits, with considerable economic loss in large production units and unfavourable prognosis for rabbits with clinical signs. The rabbits can develop health problems caused by Pasteurella, especially if they are unwell or stressed, and clinical pattern and lesions can be heterogeneous. The identification and quantification of risk factors are difficult and dependent by subjectivity of pet owner. This study is focused on pet rabbits that were diagnosed with pasteurellosis. The major stress factors identified in our study were: phonic stress, excessive manipulation, transport stress and improper feeding. Clinical and lesion data collected in this study support the heterogeneity of rabbit Pasteurella infections, previously described in several papers. The etiological study was split in following stage of diagnostics: classical bacteriological investigations and comparison of the profiles of pulsed-field gel electrophoresis bands. The study revealed that *P. multocida* strains isolated on pet rabbits have a high variability.*

Keywords: rabbit pasteurellosis, PCR (Polymerase Chain Reactions), PFGE (Pulsed-Field Gel Electrophoresis), histology, microbiology, *Pasteurella spp.*

Introduction

Pasteurella spp. is ubiquitous germ that is frequently isolated on respiratory, digestive and genital epitheliums of birds, mammals, reptiles and probably amphibians. Also, in conventional farms of domestic rabbits *Pasteurella sp.* are ubiquitous [6, 8, 10, 16, 18]. Infection most likely occurs immediately after birth and the prevalence of carrying rabbits increase to over 90% at 5 months age [6]. The stress and immunodeficiency play a major role in the onset of auto-infection for the *Pasteurella* carrier rabbits [10]. This required the development of health management programs in all holdings of domestic rabbits, adapted to the particularities of each unit in a manner that meets all the requirements of welfare.

The main pathological findings consist in rhinitis, pneumonia, otitis media, metritis, conjunctivitis, orchitis, subcutaneous abscess and septicemia [8]. The variability in clinical signs and the disease course is influenced by different bacterial virulence factors such as capsule, fimbriae, neuraminidase etc. [6]. *P. multocida* comprises 5 capsular serogroups and 16 somatic serotypes. Previously studies of capsular *P. multocida* typing performed on rabbit

isolates revealed that the main rabbit strains are type A. However, capsular type D and F can be isolated in rabbit pasteurellosis [2, 4, 13].

In veterinarian medical practice, the diagnosis of rabbit pasteurellosis is based on clinical symptoms, isolation instead cultivation or serological testing. Although the isolation and identification of the pathogenic agent represent the most reliable demonstration method of its existence in the sample that makes the object of the study, the method requires a long period and is expensive [11]. The typing of *P. multocida* by polymerase chain reaction can be a much more sensitive, specific and rapid method of diagnostic [4] if the DNA fragment amplified is from a bacterial gene involved in pathogenicity.

Nevertheless, the data supplied by PCR have values only in diagnostic, and one of the usually question of pet owner is that the rabbit disease is hazardous for his family. The problem is more delicate if in the same time with rabbit pasteurellosis in the owner family was a similar pathological event. *Pasteurella* species, particularly *P. multocida*, have a worldwide distribution and different strains can be involved in same herd or epidemiological unit. In conclusion a simple isolation of *P. multocida* in rabbit and human is inconclusive. Boerling P. et al. proposed a molecular identification and epidemiological tracing of *P. multocida* in case of humane baby meningitis. An 1-month-old baby with *P. multocida* meningitis was exposed to close contact with two dogs and one cat. The source of infection was clearly confirmed with macrorestriction fingerprints (generated by PFGE) when the same strain of *P. multocida* subsp. *septica* was demonstrated as being present in the cat tonsils and the baby cerebrospinal fluid [1]. Unfortunately, dogs and cats are not the only pet able to transmit this infection, and occasional cases of pasteurellosis in humans from laboratory rabbits were reported [3]. In this context, the role of rabbit pets in some epidemiological event need to be considered. In our opinion the comparative analysis by macrorestriction fingerprints of *Pasteurella* strains isolated in human or animal outbreaks can be extended to all companion animals and the development of a macrorestriction fingerprints database can be a good start. Also, it may be used in epidemiological investigation of *Pasteurella* infections into rabbit herds or rabbits supplied by same pet shop.

In this paper the main object is evaluation of *Pasteurella multocida* strains heterogeneity in some Romanian rabbit outbreaks of pasteurellosis by PFGE, a diagnostic method usually used in molecular laboratories.

Materials and methods

In this paper we focus on the relationship between *Pasteurella* pathology and rabbits as companion animals. We present 8 outbreaks of rabbit pasteurellosis, and 37 animals with or without clinical signs involved in those events. All the rabbits that made the object of the research were assessed at the University Hospital of the Faculty of Veterinary Medicine, Bucharest. The rabbits were from Bucharest and surrounding suburbs. Information on the outbreaks, such as number of animals, clinical symptoms, age, gender and breed is available upon request. All investigated animals were not vaccinated. The methods used in clinical and post-mortem investigations were described previously [17, 23, 24].

Table 1. Number of animals, clinical symptoms, age, gender and breed of rabbits infected with *Pasteurella* sp.

Outbreak	No. of rabbits	Gender		Breed	Age (weeks)	Clinical symptoms						No. of <i>Pasteurella</i> Isolates	Site of isolation
		♀	♂			Rhinitis	Conjunctivitis	Otitis media	Subcutaneous abscess	Metritis	Pneumonia		
I	2	1	1	Angora	4 <	+	+	-	-	-	+	1	nose/ lungs
II	6	4	2	Chinchilla	6-12	+	-	-	-	-	-	1	nose
III	10	7	3	Chinchilla	-	+	+	+	+	+	+	2	nose/ lungs
IV	2	1	1	Chinchilla	7	+	+	-	-	-	+	1	nose/ lungs
V	3	2	1	Angora	9	+	+	-	-	-	+	1	nose/ lungs
VI	8	5	3	Chinchilla	-	+	+	+	+	+	+	2	nose
VII	4	2	2	Chinchilla	15	+	+	-	-	-	+	1	nose/ lungs
VIII	2	1	1	Angora	6	+	+	-	-	-	+	1	nose/ lungs
Total	37	23	14			+	+	+	+	-	+	10	

Before the sampling for bacteriological diagnostic, relevant epidemiological information was obtained from the owners of the companion rabbits. After clinical examination, from the animals with rhinitis (29/37) and the clinically healthy animals (8/37) were collected nasal discharges with sterile cotton swabs. Additional, from all necropsied animals (13/37) were collected pulmonary secretions with sterile cotton swabs. In the outbreaks II (6 rabbits) and VI (8 rabbits) the necropsy was not performed because the bodies were improper stored (the advanced state of deterioration). Nasal and lung cotton swabs were preserved on the Amies medium.

Histopathological investigations were focused to evaluate the pulmonary lesions. 13 pulmonary fragments with macroscopic lesions were suspended in 10% neutral buffered phosphate formalin. The sections were embedded in paraffin, cut at 5µm and hematoxylin-eosin stained.

The methods used in bacteriological diagnostic were described previously [11]. Briefly, the specimens were dispersed on agar with 5% defibrinated ram blood medium (Cantacuzino Inst., Romania), AABTL medium (Cantacuzino Inst., Romania) and on MacConkey agar medium (Merck, Germany). For differentiation of *Pasteurella* species and subspecies polytropic media (Cantacuzino Inst., Romania) were used for testing the acidification of three sugars: glucose, lactose and saccharose and the H₂S production – and MIU – means of which the motility of the germ and the indole production and urease are tested. The acidification of carbohydrates was identified by seeding in tubes with a methylene blue indicator, using media prepared by the “Cantacuzino” Institute: arabinose, glucose (for testing the gas production), mannitol, sorbitol, trehalose and D-xylose; the carbohydrate media were incubated at 35-37°C, for 4 days. Other tests included the ornitin decarboxylation and the urea hydrolysis (Christensen medium).

It was evaluated the non-hemolytic aspect after incubation on the blood-agar medium, to the presence of Gram-negative, non-motile coccobacilli, with positive test for indol, catalase and oxidase, and to the strains which produced acid from glucose and saccharose and which were lysine-decarboxylase negative.

Evaluation of *Pasteurella multocida* pathogenicity was performed on 5-week-old conventional mice (5 animals/test) by intra peritoneally injection of 0.2 ml of 18h broth culture.

For the macrorestriction analysis of *P. multocida* strains, PFGE was carried out according to Ivana S. et al. [11]. Briefly, a bacterial suspension in Tris-EDTA buffer of *P. multocida*, cultivated on gelose with 5% ram blood (4U McFarland, $\approx 10^9$ CFU/ml) used for the DNA preparation. The cell lysis was performed in a lytic buffer containing 50 mM tris, 50 mM EDTA, 1% sarcosil and 0.5 mh proteinase K. DNA was digested with 40 U *ApaI*. The DNA fragments which resulted were separated by a CHEP Mapper (Bio Rad) system in 1% agarose SeaKem Gold gel. The electrophoresis conditions were the following: Tris-Borat-EDTA 0.5x buffer, at 14°C, 6V/cm of electric field, an angle of 120° and a linear ramp. For the separation of the macrorestriction *ApaI* fragments migration programme lasted 20 hours, with a pulse spacing of 1-30s.

Results and discussions

The epidemiological records and clinical data collected in all outbreaks of rabbit pasteurellosis (table 1) have a great value in risk analysis of *P. multocida* incidence. The contamination with *Pasteurella* spp. was identified in all age and gender categories. In our study, the increased number of females was correlated with the increased number of females investigated, and the linear correlation was irrelevant. In concordance with literature reports [3, 8, 18], in all eight outbreaks the young animals were more frequent involved in infections with clinical manifestation.

The case history of first outbreak revealed the following deficiencies in housing and feeding conditions:

- improper rabbit hutch: a small cat travel cage without hay or other type of bedding and an instable improvised water-bowl;
- poor quality of feeding: bretzel, biscuits;
- the rabbit cage storage in the living room, near the home cinema system speakers;
- the rabbits were transported to veterinary clinic in the car trunk (45-50 minutes/route), in condition of urban traffic.

The case history of second outbreak revealed the following deficiencies in housing and feeding conditions:

- improper rabbit hutch: hosting in a glass cage, with poor ventilation in last week before start of outbreak;
- the glass cage was built into small temporary exhibition hall, near to main access road;
- the rabbits were transported daily from the owner house to the exhibition hall.

The case history of third outbreak revealed the following deficiencies in housing and feeding conditions:

- improper rabbit hutch: rabbits of different ages and sizes housed in a small cage;
- excessive manipulation of rabbit by several people interested in buying a pet;
- improper feeding: periodically rabbit received from children bread, bretzel or biscuits;
- transport stress: the rabbits were transported daily from the owner house to the temporary livestock market.

The case history of fourth outbreak revealed the following deficiencies in housing and feeding conditions:

- improper rabbit hutch: small cage without water supplier;
- excessive manipulation by owner;
- improper feeding: only lettuce;

- transport stress: in last week the rabbits were transported daily to veterinarian.

The case history of fifth outbreak revealed the following deficiencies in housing and feeding conditions:

- improper rabbit hutch: a small cat travel cage without hay or other type of bedding and an instable improvised water-bowl;
- excessive manipulation by the owner children;

The case history of sixth outbreak revealed the following deficiencies in housing and feeding conditions:

- improper rabbit hutch: the cage built around a heavily traveled road;
- excessive contact and manipulation by children;

The case history of seventh outbreak revealed the following deficiencies in housing and feeding conditions:

- improper rabbit hutch: hosting in a glass cage;
- excessive contact and manipulation by children;
- poor quality of feeding: bretzel, biscuits.

The case history of eighth outbreak revealed the following deficiencies in housing and feeding conditions:

- improper rabbit hutch: a small cat travel cage;
- poor quality of feeding: bretzel, biscuits;
- the rabbit cage storage on the kitchen floor;
- the owner has two small sized dogs that bark frequently at rabbits.

The case history is a key in analyzing of each pathological event in rabbit pasteurella carriers. In the context of the sub-clinical carriers of *Pasteurella*, the pet rabbits stressed by an excessive handling and an improper accommodation (noise, poor housing, frequent transport, poor diet) have a great risk to develop a grave form of pasteurellosis by auto-infections. In fact, the rabbits are particularly sensitive to environmental stressors and handling, and those stressors are usually associated with the clinical expressed pasteurellosis on rabbit.

The etiological diagnosis was obtained after death of all ill rabbits. The evolution of disease was peracute/acute and the therapeutical actions were limited. Also, in some rabbit family were identified asymptomatic infected animals (II, III, and VI). Usually, rabbit pasteurellosis was clinically manifested by rhinitis. Symptomatology consists in sneezing, coughing, nasal serous, white or purulent discharges and noisy breathing. Peracute septicemia with sudden death was not characterized by nasal discharge. This type of pasteurella infection was frequently described in young pet rabbits. In acute form the pasteurellic pneumonia was associated with rhinitis, fatigue, lethargy, depression, anorexia and progressive weakness.

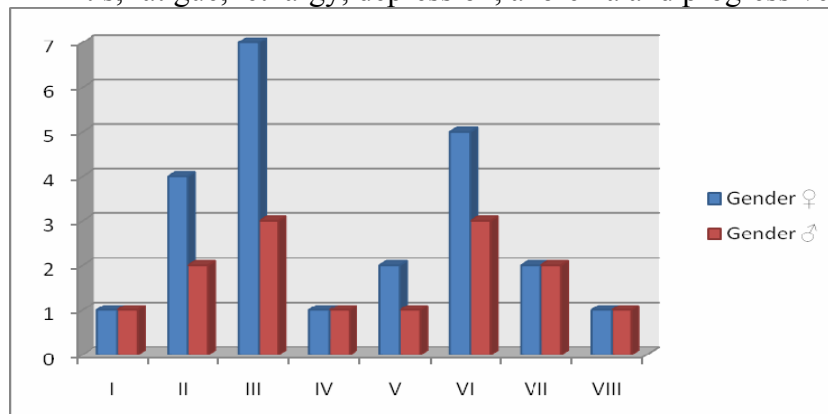


Figure 1. Gender proportion in eight rabbit pasteurellosis outbreaks. In evaluation of the gender – disease correlation is important to have an similar proportion of both genders. In this study 62% of rabbits were female, and all rabbit were infected.

Usually, the diagnostic of lower respiratory tract disease is based on clinical exam and X-rays. Often the trachea and lungs listening permit the identification of the place where the airflow is obstructed. X-rays confirm the thoracic disease, and can differentiate the origin of respiratory signs. Thoracic ultrasounds can determine if abscesses or densifications are present. Blood tests are not useful in determining the etiology, but may be made in any context to determine the metabolic profile and evaluating the presence of concurrent disease. *Pasteurella* serological testing can be performed, but results are difficult to interpret [2].

In lower respiratory tract the acute/subacute forms of pasteurellosis, with a history of over 5 days, are associated with marked pulmonary consolidation, often with hemorrhagic and fibrin-purulent exudates in affected areas. Pulmonary lesions are frequently accompanied in sub-acute forms by pleuritis, fibrin purulent pericarditis and rarely with empyema [12, 17, 19]. Acute-subacute forms are histopathologically characterized by heterophilic and macrophage infiltration, and a marked alveolar interstitial infiltrate, but the airways are relatively free of inflammatory exudates. In some cases the cell population that invade lung can be represented by polymorphonucleated, this cellular infiltration is associating with pleural fibrosis. The rabbits that will die at 7 days post-infection develop fibrin purulent bronchopneumonia with varying degrees of enlargement, while those that die at 9 days post-infection showed a marked congestion of alveolar capillaries and pulmonary edema with protein material [19]. In our study the histopathological findings were similar to these described in literature. The histological findings were used in evaluation of etiological role of *Pasteurella* strains isolated from the rabbits when the bacteriological exam revealed presence of several pathogens.

Clinical and lesion data collected in this paper, and the information provided in many previous works with same subject supporting the heterogeneity of rabbit *Pasteurella* infections [5, 6, 8, 10, 12, 16, 18, 19].

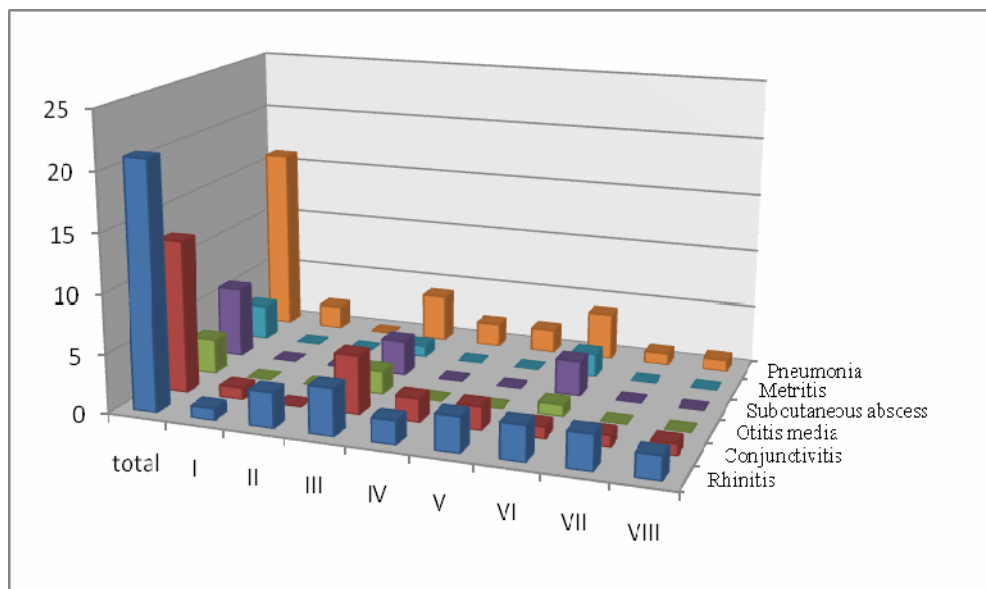


Figure 2. The rabbit pathology associated with *P. multocida* infection. The rabbit pasteurellosis is frequent associated with rhinitis, but pneumonia and conjunctivitis are also described in many outbreaks. Therefore, the sampling protocol used for laboratory diagnostic has an increased success by using the swabs that collect the nasal or pulmonary secretions. This observation creates premises for the best samples use in rapid diagnostic of rabbit pasteurellosis by PCR techniques from clinical samples.

Special problems in diagnosis are found in peracute/acute pasteurellosis of young pet rabbits. The small number of pet rabbits, without signs or with moderate signs often required

extensive investigations for confirmation, and that can delay therapeutic intervention and thus reduce the chances of recovering the animal. The lesions are not always helpful, often those have septicemia character. In addition, *Pasteurella* is not the single leporidae respiratory pathogen. *Bordetella bronchiseptica* is also involved in respiratory pathology of rabbits, *Klebsiella pneumonia*, pneumococcal and virus infections may have respiratory symptoms. In this context, we decided that if the *Pasteurella* will be associated with one or more bacteria, the bacteriological protocol will be extended to identification of all isolated. For this study were selected only the outbreaks when the *Pasteurella* pathogenicity was involved in pathological event.

The following characteristics of *Pasteurella* spp were demonstrated: coccobacilli or small rods with Gram negative bipolar staining, microaerophilic, non-hemolytic, non-motile, capsules, without growth on the Mac Conkey agar, oxidase-positive and saccharose fermentative coccobacilli, without gas production. Colonies had the usual characteristics. Other biochemical properties included the indole reaction, the catalase reaction, the nitrate reduction, the ornithine-decarboxylase and the urease production.

The bacteriological investigation revealed ten *P. multocida* strains, one *Klebsiella pneumonia* strain, one *Staphylococcus* spp strain, four *Escherichia coli* strains, two *Streptococcus* spp strains and one *Pseudomonas aeruginosa* strain.

The following results were obtained from pathogenicity tests on mice:

- the strains from outbreaks I, II, IV, VII and VIII and one from outbreak III killed all mices in one day;
- the second strain from outbreak III, killed mices in 1- 2 days;
- the strain from outbreak V killed mices in 1- 3 days;
- one strain from outbreak VI killed mices in 1- 2 days;
- one strain from outbreak VI killed mices in 1- 3 days;

The pathogenicity tests results revealed the main role of *P. multocida* in pathological events described in this paper, the variation of *P. multocida* strains virulence and the potential risk of sub-clinical *Pasteurella* carrier rabbits to develop systemic infections. The results are in concordance with previously studies [6, 20], and justify the following step of investigations: macrorestriction fingerprints generated by PFGE.

Pulsed-field gel electrophoresis (PFGE) is a genetic typing method that is widely used as a molecular epidemiological tool for studying the genetic diversity of numerous bacterial pathogens.

In this study the typing method by PFGE was chosen as the “gold standard” of bacterial typing methods. PFGE was found to be a very accurate method in bacterial strain typing [22].

Intact bacterial cells are embedded in soft agarose plugs followed by lysis of the cell wall *in situ* to minimize shearing of the chromosome. Owing to the high discriminatory capacity of PFGE, results may be obtained which can elucidate the equivocal situations occurring in the classical epidemiological investigation, pointing out the heterogeneity/clonality of bacterial isolates.

The profiles of bands generated by *ApaI* in the restriction analysis of chromosomal *P. multocida* DNA were with a lower number of bands, well separated, being well integrated in the interval of development of the lambda markers. Taking into account the good discrimination between isolates based on the clarity of the profiles of *ApaI* generated bands, a correct interpretation of the genetic affinity degree was possible. The restriction analysis revealed ten different profiles (PFGE-types) among all *P. multocida* isolates from rabbit (figure 3). The comparison of the profiles of PFGE bands by means of Fingerprint II

programme and their interpretation on the basis of the Dice coefficient and of the UPGMA analysis method revealed a heterogeneous *P. multocida* population.

The similar data were reported when the restriction analysis with *ApaI* were performed in pig *Pasteurella* respiratory infections; Lainson F.A et al found fifteen electrophoretic types were identified among the 51 isolates examined from one cohort [14]. Also, the DNA fingerprinting by PFGE of avian [25] and feline [11]. *P. multocida* isolates revealed that it is useful for accurate identification and epidemiologic study of *P. multocida* isolates. All this data supporting the opportunity in development of *P. multocida ApaI* profiles database that will include besides pathogen strains of *P. multocida* isolated from farm animals and pet animals. By this database will be more easy to identify and tracing pathological events with or without zoonotic implication.

Unfortunately, in our study the analyses of *P. multocida* clonal characteristics were limited at the assessment of the polymorphism degree of strains circulating in companion rabbit population, without the existence of any epidemiological relationship between the investigated isolates and strains isolated from other animal and human species.

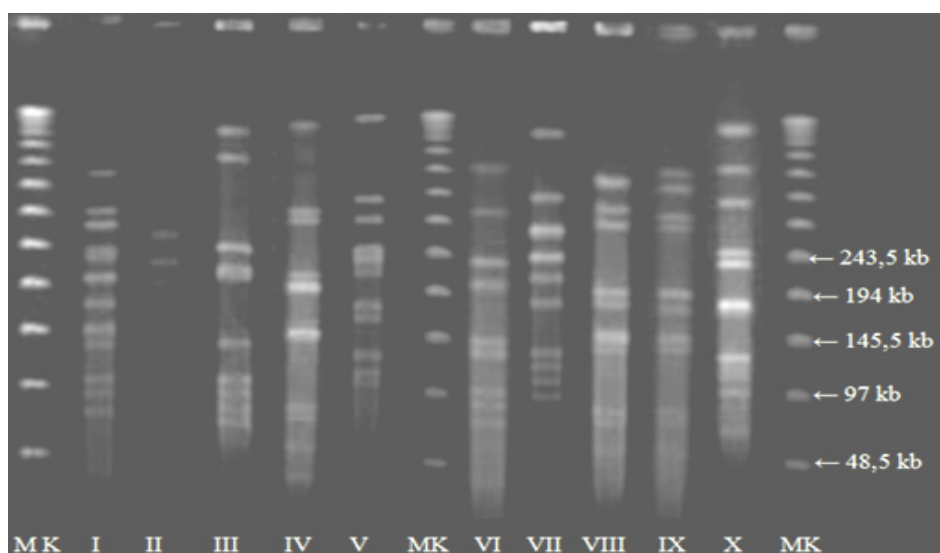


Figure 3. The profiles of PFGE bands showed a significant heterogeneity of *P. multocida* isolates from rabbit (I-X = *P. multocida* rabbit isolates)

In conclusion, the profiles of PFGE bands showed a significant heterogeneity of *Pasteurella* isolates. Therefore, the biodiversity of *P. multocida* rabbit isolates create difficulties in spatial and temporal dynamic evaluation of each strain if the typing of each isolate is not performed. PFGE has been shown to have a great discriminatory power in the typing of *P. multocida* rabbit isolates and the method can be used as epidemiological tool in rabbit pasteurellosis study.

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