

## CHARACTERIZATION OF *PASTEURELLA MULTOCIDA* RECOVERED FROM LIVE RABBITS AT A SMALL-SCALE FARM PREVIOUSLY MANIFESTING DEATHS BY PYOTHORAX AND PYOMETRA

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### ABSTRACT

Long distance transport and relocation of adult rabbits into another small-scale farm was shortly followed by mating to the bucks of the same group. Within a 6 weeks period an outbreak of the respiratory syndrome occurred amongst these animals. Mortality of pregnant and nursing females, and their pups was 50% and 70% respectively. Main pathomorphological lesions included pyothorax, pleural empyema, and pyometra or necrotised foetuses. The aboriginal rabbits of the farm remained healthy at the same period. Nasal and vaginal colonization with *P. multocida* was monitored on live rabbits of the relocated group and of the local animals soon after the outbreak. Proportion of the nasal carriers was 70% in the relocated group and that was around three times higher compared to the value obtained at the group of local rabbits. Vaginal colonization was detected at 33% of the females in the relocated group and each of vaginal positive females was nasal positive either. *P. multocida* isolates were genotyped with REP-PCR and relatedness of the products obtained with each isolates as template were calculated. The result showed that the strains originated from the recently introduced animals were clearly different from that of the local population. Strains isolated from the nasal and vaginal mucosa were different within a rabbit, and clustered together by other strains according the anatomical region of the isolaton. REP-PCR was proven to be highly discriminative beside its velocity and simplicity in characterisation of *P. multocida* isolates. Based on the results of the genetic analysis the most probable cause of the outbreak is a *P. multocida* strain that colonized the nasal mucosa of the mounting buck, and was carried from its original stock. Furthermore the severity of the outbreak could be influenced by the relocation as stress factor.

**Key words:** pasteurellosis, REP-PCR, nares, vagina, environmental stress, pathomorphology.

### INTRODUCTION

Field reports describing pneumonia, pleural empyema, otitis media and subcutan abscesses of rabbit caused by *Pasteurella multocida* have been reviewed by Di

GIACOMO (1992). Most of those papers concern chronic infections, which are causing sporadic losses. However, sudden activation of latent infection in larger number of carriers at the same time can lead to more severe consequences. Number of publications about this kind of outbreaks is very few.

PATTON (1990) listed the most important factors resulting the culling of female rabbits and discussed how pasteurella infections could stay behind low reproductive performance of does. ROSELL *et al.* (1992) found that 95% of does died by respiratory pathological changes had have reproductive disorders also. By the current opinion pneumonia, otitis, metritis etc. are different clinical forms of the respiratory syndrome and could stem from rhinitis by infection descending to different organs via blood circulation and lymphatic system (ROSELL *et al.*, 1992; PATTON *et al.*, 1984). Vaginal status of breeding females considering *P. multocida* colonisation to detect ascending route for uterine infection or vertical transmission to pups were evaluated only by HOLMES (1983) and PATTON *et al.* (1984).

Latest results in bacterial molecular biology gave the possibility for genotyping pathogenes and following their spread. REP-PCR process was developed by VERSALOVIC *et al.* (1991) based upon repetitive sequences present in the bacterial genome. TOWNSEND *et al.* (1997) with this technique detected strain-to-strain variation of *P. multocida* isolates causing haemorrhagic septicaemia. The great discrimination that can be achieved by this type of genotyping makes it reliable tool for epidemiological studies.

This study reports on an outbreak of the pasteurellosis following the transfer and resettlement of rabbits into another rabbitry, and which remained restricted to these rabbits and did not appear within the indigenous population of the farm taking them. Bacterial genotyping allowed to differentiate *P. multocida* strains isolated from the nasal and vaginal mucosa of rabbits recently introduced and that of the local rabbit population.

## MATERIAL AND METHODS

### Description of the involved farm

The small-scale commercial farm had 80 does and breeding replacement was performed by locally reared females. Does were mated to bucks of the same population. Five weeks before the outbreak 18 female and 2 male rabbits were introduced into the rabbitry. The place of their origin has not got any previous relationship with the rabbitry under study. The geographical distance is more then 800 km between the two places. The newly arrived rabbits also belonged to a different breed.

Following their arrival these animals have been settled separatedly in a hutch apart and females were mounted by the bucks arrived with them until became pregnant. When the time of parturition arrived they were transferred into another building equipped with nestboxes and placed amongst locally reared does.

### **Sampling**

Samples were taken by inserting sterile cotton tipped swabs 2-3 cm deep into the external nares or into the vagina.

Nasal and vaginal swabs were collected from 9 females and 1 buck belonging to the newly introduced group of animals, does being not pregnant to the moment of sampling. Further nasal samples were collected from 5 broilers of the local population. Four swab samples were collected from the inside environment (board, wall, nipple and feeder surface) of one cage.

### **Bacterium isolation**

Swabs were spread to sheep blood agar plates within 3 hours, and cultured for 24-48 hours in a humidified thermostat at 35°C. Mostly mixed cultures growing on BSA plates were presumably identified as *P. multocida* carriers when 0.5-1 mm size whitish-grey, translucent and indole positive colonies have been detected. Single colonies were then picked up and subcultured to dextrose starch agar and MacConkey agar and incubated for 24 hours at 37°C. The isolates which were Gram-negative rods that produced indole, were oxidase and catalase positive and failed to grow on MacConkey agar, and were positive by the diagnostic PCR developed by TOWNSEND *et al.* (1998) have been identified as *P. multocida*.

### **Genotypic characterisation**

REP-PCR had been performed as described by TOWNSEND *et al.* (1997). For ease and rapidity 2-3 colonies were picked up from plates containing pure cultures of *P. multocida* as template. The PCR mixture contained 1× PCR buffer, 4 mM MgCl<sub>2</sub>, 200 μM of each of four dNTP, 2 U Taq DNA polymerase (PROMEGA Life Science) and 1 μM of each opposing primer (VERSALOVIC *et al.*, 1991) in 50 μl final volume. Amplification reaction was performed in a TPersonal (Whatman Biometra) thermalcycler. Cycling conditions were as follows: one cycle for 7 min at 95°C, 1 min at 42°C, 8 min at 65°C; 32 cycles for 1 min at 94°C, 1 min at 42°C, 8 min at 65°C; and a final extension for 8 min at 65°C.

REP-PCR products were electrophoresed at 2.5 V cm<sup>-1</sup> for 3 hours on 1.5% agarose gel in 1× TAE. DNA fragments were visualised by UV transillumination and photographed.

### **Statistical evaluation of strain relatedness**

Analysis of DNA fingerprint considering fragments between 200 and 3000 bp were performed using a BIO-1D software (Wilber Lourmat). Lanes that were blank because the PCR failed and lanes with limited number of bands were not included in the analysis. The images were normalized using the 10 kb ladder as an external reference standard. REP-PCR pattern similarities were quantified by calculating the Dice coefficients with 6% of confidence and constructing a dendrogram from the resulted data using the UPGMA clustering method.

## RESULTS AND DISCUSSION

### Field pathology observations

The outbreak regarded only to the recently introduced animals, meanwhile within the local population no symptoms of respiratory syndrome were discovered. Nine females and one buck died in a two-week-long period (50% mortality). These does were all pregnant and 6 died just some days before their scheduled parturition. Necropsies discovered pyothorax, pleural empyema, and pyometra or necrotised foetuses. The remaining females delivered but 70% of their sucklings died between 10-17 days of age. In these pups the main pathomorphological lesion was pyothorax.

At the day of sampling 5 of 10 swabbed animal was healthy looking, 3 had rhinitis, 1 had subcutaneous abscess in the group of newly arrived rabbits. One more female of the same population was in bad condition and had pododermatitis on four legs.

Of the broiler rabbits belonging to the local population 3 presented rhinitis, conjunctivitis or subcutaneous abscess, and two had light body weight at 12 weeks of age but without sign of disease.

SMITH (1927), ALEXANDER *et al.* (1952), and SATO *et al.* (1967) have reported outbreaks of pasteurellosis resulting high mortality between breeding animals, comparable to that what was found here. The factors leading to such an outbreak have not been discovered, except at SMITH (1927) when mixing of susceptible and infected populations resulted in local epizootic.

At the farm reported here, already infected animals died of respiratory syndrome after having been exposed to stressing environmental factors by lengthy transport and relocation what was immediately followed by mating and pregnancy. CAMPS (1976), later LOLIGER and MATTHES (1989) and KPODEKON *et al.* (1999) have emphasized the role of exogenous secondary effects in exacerbation of the pasteurella infection. Nevertheless PAPP *et al.* (1989) were not able to provoke epizootic outbreak in infected rabbit population experimentally exploring the animals to elevated NH<sub>3</sub> level, cold or warm temperature. Recently climatization of rabbit houses provides optimal environmental conditions but relocation of the animals occurs more and more often as hybrid breeding extends.

Respiratory mortality between suckling rabbits appeared only at around 8% in an epidemiological survey performed by ROSELL *et al.* (1992). Eventually this value can increase to higher level when females are involved in an outbreak like in the case detailed here.

### Bacteriology results

All nasal swabs were positive for aerobic bacterial growth, 6 of 9 (66%) vaginal samples however were negative. *P. multocida* were isolated from 1 of 5 and 7 of 10 nasal swabs in the group of aboriginal or just brought in animals, respectively. All bacteria positive vaginal swabs yielded *P. multocida* isolates. No *P. multocida* was detected at the inside environment of the cage. Clinical observations did not seem to have relation with the *P.*

*multocida* carrier state whereas 2/5 and 6/10 of asymptomatic and ill animals respectively presented infection.

The difference (50%) in the presence of nasal *P. multocida* carriers in the two groups could be referred either to the different origin and breed of the rabbits or to the negative environmental factors affecting only the relocated group of rabbits. Prevalence in each group is within the range detected at earlier studies (reviewed by DIGIACOMO, 1992 and by KPODÉKON *et al.*, 1999).

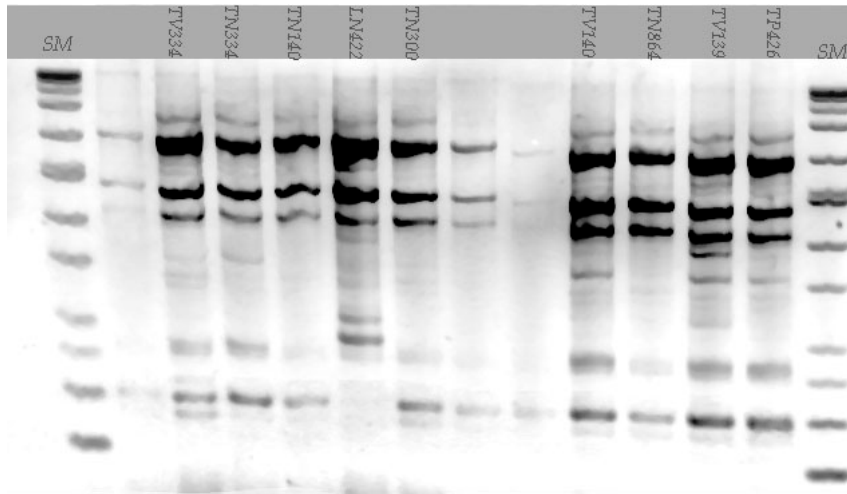
Colonization of vagina was investigated only at few cases. JAKUES *et al.* (1986) studying normal microflora of the genital tract of female rabbit failed to recover *P. multocida*. The incidence of vaginal *P. multocida* in a commercial rabbit population was 25% (HOLMES *et al.*, 1983), and 78% of vaginal positive females were nasal positive also. In the study presented here 33% of vaginal samples was positive for *P. multocida* and each of vaginal positive females were nasal positives either. On the other hand PATTON *et al.* (1984) did not cultured *P. multocida* from the vagina of nasal positive does rearing their also positive newborn litters. That could lead to the conclusion that vagina and the litter are infected by her nasal discharge when cleaning up itself or at visits of the litter, respectively.

MCKENNEDY and SHILLINGER (1938) isolated *P. multocida* from the vagina of only 9% of breeding does which have been mated to the same buck and developed thereafter acute septicaemia or purulent discharge from the reproductive tract. At the outbreak observed herein the vaginal positive does have been mated to the buck that died later in suppurative pneumonia, but stayed empty. Sample from that male was missing, so it's state remained unknown.

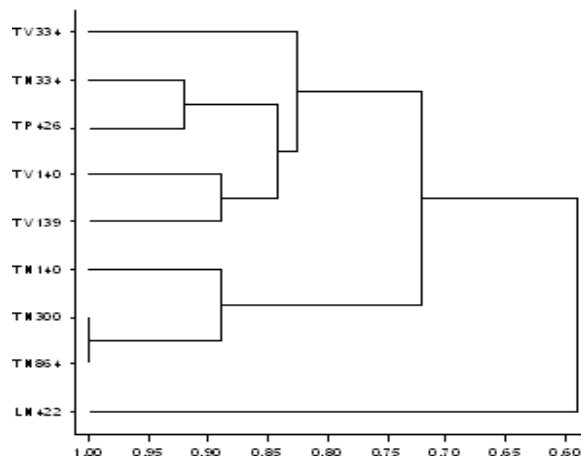
### **Genotypic characterisation**

Fragments generated by REP-PCR ranged from ~300 to ~2800 bp in size in all isolates, and produced complex banding pattern shown in fig. 1. The very definitive band slightly below 1400 bp being present at every isolates might indentify *P. multocida ssp. multocida* (CHEN *et al.*, 2002).

On the dendrogram (fig. 2) three major clusters were observed. Cluster I was observed at 60% similarity and this contained only one isolate, coming from the local population. Cluster II observed at 73% similarity comprised three isolates, all obtained from the nasal mucosa. Cluster III were composed from five isolates including all non nasal (vaginal mucosa and pododermatitis) samples being similar at 83% level.



**Figure 1.** Gel of REP-PCR products from different isolates. In the labels L= local, T= transported; N= nasal, V=vaginal, P=pododermatitis; the number= animal's identity. Lanes 1 and 14 represents molecular size markers containing 200, 300, 400, 500, 750, 1000, 1400, 1500, 2000, 3000, 4000 and > 5000 bp bands (Sigma). Lanes without label were not included in the evaluation.



**Figure 2.** Dendrogram with homology coefficient at 6% confidence (UPGMA) showing relatedness of *P. multocida* strains isolated from nasal (N) and vaginal (V) mucosa of rabbits (ID number) belonging to the local (L) or to the recently arrived (T) groups.

Considering relatedness of the isolates, the only one isolate from the group of local rabbits (LN422) clearly clustered apart from the others, showing 40% dissimilarity. This strengthens the idea that the outbreak might be caused by a *P. multocida* strain that has originated on the distant farm and was dragged by the newly introduced rabbits themselves.

The isolates cultured from the recently brought in rabbits grouped into two subclusters, mostly nasal samples and mostly vaginal samples together, respectively. Even *P.*

*multocida* strains isolated from the nasal and vaginal mucosa of the same animal (TN140 and TV140) were assigned to different clusters, with only 75% similarity. At the same time nasal isolates of three different animals (TN864, TN300 and TN140) and vaginal isolates of two animals (TV139 and TV140) had 90-100% similarity and could be considered as identical. This can be explained as strains included in the two clusters were acquired by different routes and from different animals. Nasal mucosa is easily colonized when carrier animals are in the same area and bacteria are transferred by immediate contact or by fomites. Colonization of the vaginal mucosa can be done by the doe when cleaning itself, beside this also by the course of natural mating, when the buck smells the perianal region of the doe. The high level similarity of these two isolates might be a consequence that the same buck was used to mate, while the does with *P. multocida* negative vagina might have been mated right to the negative buck, or were not receptive at all.

### CONCLUSIONS

The facts that no animals had died from pasteurellosis in the aboriginal group, the prevalence of carriers was lower with 50% amongst them, and the one respective isolate was clearly differentiated by genetic analysis allow the conclusion that relocated rabbits introduced recently to the outbreak farm were carrying the *P. multocida* strains, one of which caused the deadly pasteurellosis of their farm-mates. The most probable source of vaginal colonization might be an infected male when natural mating was carried out.

### ACKNOWLEDGEMENTS

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