PHENO- AND GENOTYPIC CHARACTERIZATION OF *PASTEURELLA MULTOCIDA* STRAINS RECOVERED FROM HEALTHY AND DISEASED RABBITS

Virág Gy.[1], Barna T.[2], Fábián K.[2]*, Farsang A.[2]

[1]Research Institute for Animal Breeding and Nutrition, Isaszegi ut 200, 2100 Gödöllő, Hungary
[2]Central Agric. Office, Directorate of Veterinary Medicinal Products, Szállás u. 8, 1107 Budapest, Hungary
*Corresponding author: fabianka@oai.hu

ABSTRACT

The characterized 32 *Pasteurella multocida* isolates were collected through 2-years long period, taken from rabbits in the fattening and the breeding units of a commercial rabbit farm. Rabbits were manifesting of (n=18) or free from (n=14) signs of *pasteurellosis*. All isolates were identified with standard microbiological tests and *P. multocida* specific PCR. After 24 hours incubation at 37°C on TSA, 72% and 28% of isolates developed large or small colonies, respectively. Colony type was smooth (n=17), mucoid (n=13) and rough (n=2). Capsular typing was performed with PCR and presence of *capA* and *F* genes were detected for 53% and 28% of strains respectively. Occurrence of gene *capD* was only 9%. Based upon carbohydrate fermentation results, four distinct biochemical types could be recognized: biovars 1 (25%), 3 (6%), 6 (44%) and 9 (3%). Further 22% were non typeable isolates representing lactose positive variants of biovar 2 and 6. An association between capsular type and biovar could be supposed, as biovar 1 strains (n=8) possessed exclusively *capA* capsule gene. Biovar 6 (n=14) and unassigned (n=7) strains had any capsular types. The colony size, biovar and capsular type did not show association with the rabbit sanitary state. Similarity matrix was calculated from the fermentation results of all 50 carbohydrates included in the API CH50 test strip. One group containing five quite diverse isolates taken only from animals with pathological signs appeared on the dendogram and another two pathologic isolates were also separated as single member clusters, each differentiated around 40% similarity level. One further cluster included 7 of 9 capsular type F strains above 90% within group similarity, which were isolated within a very short time interval, and 5 from healthy rabbits. REP-PCR genotyping revealed most strikingly more pairs of genetically identical strains, each of both isolated from diseased or from healthy rabbits, or one from diseased and another one from healthy rabbits. Clustering otherwise did not show obvious association of the genotype with biochemical or serotyping characteristics of the isolates nor with the sanitary state of the sampled rabbit. The conclusion is that high phenotypic and genotypic diversity is characteristic to the *P. multocida* strains isolated from the same herd across time. Higher similarity of biochemical traits presented by isolates collected within a short period is not inevitably associated with higher pathogenecity.

Key words: *Pasteurella multocida*, Sanitary status, Rabbit, Biovar, Serogroup, Genotype.

INTRODUCTION

Rabbit females are frequently carriers of *Pasteurella multocida* without or with recurrent lesions or decreased production. Despite that significant variations in the phenotypic properties of *P. multocida* isolated from rabbits have been reported (Chengappa *et al*., 1982; Percy *et al.*, 1984; Badiola *et al.*, 1996) comparison of strains recovered from rabbits which are free from or manisfesting signs of *pasteurellosis* have been scarcely made (Lu *et al*., 1978 and 1983) till now. Genetic diversity of rabbit *P. multocida* isolates have been detected with the recently introduced repetitive extragenic palindromic sequence polymerase chain reaction (REP-PCR) genotyping by Cerrone *et al.* (2005). All of these studies were performed on isolates collected from different farms and mainly from pathological sources.

The aim of this study was the characterisation of *P. multocida* isolates collected in one commercial rabbit farm according to the presence or absence of *pasteurellosis* lesions on the rabbit and comparison of pheno- and genotypic variation amongst these.

MATERIALS AND METHODS

Animals and sampling

Through a two years period (2002 autumn – 2004 autumn) the prevalence of *P. multocida* was monitored in breeding and broiler rabbits in propagation (Ócsa, Hungary) and fattening (Lajosmizse, Hungary) units of the commercial rabbit farm involved. Hygienic and sheltering conditions matched the standard requirements. Point prevalence of *P. multocida* detected at different sampling occasions was between 1.5% and 60%, occurence of *pasteurellosis* outbreak however was not evident at any time. There was a high carrier rate and the disease was present mainly in its chronic forms. Sampling was performed on clinically healthy rabbits with good production level, on live rabbits manifesting typical signs of *pasteurellosis* (rhinitis, torticollis, subcutaneous abscess) and from corpses of rabbits succumbed to *pasteurellosis*. Bacteria were collected from nasal mucosa of live rabbits or from lung, pharynx or subcutaneous abscess at autopsy of corpses by sterile cotten-capped swabs. Swabs were carried to the lab at 5°C and plated within 4 hours of collection. Twenty nine isolates were chosen for detailed characterization, 12 of which came from live and clinically healthy rabbits, 7 from live rabbits apparently suffering from lesions typical to *pasteurellosis* or with weak performance by other causes, and 10 from died rabbits.

Isolation, identification, pheno- and genotypic characterization

Swabs were spread onto SBA plates and incubated at 37°C for 24 hours. Suspected *P. multocida* colonies were subcultured on TBA and McConkey plates and incubated at 37°C for 48 hours. All culture media were prepared with chemicals from *Scharlau, Barcelona, Spain*. Isolates were identified with standard microbiological tests and *P. multocida* specific PCR (Townsend *et al., 1998*). Spot indole, oxidase and catalase tests (Merck, Darmstadt, Germany) were performed on bacterial colonies taken from the plates. ODC test was performed in DLB broth according to Falkow. Fermentation of 50 carbohydrate substrates was detected on API-CH50 test strip (Biomerieux, Marcy l'Etoile, France). Capsule genes *capA, D, F* and genomic DNA between repetitive extragenomic palindromic (REP) sequences were amplified by PCR performed as described by Townsend *et al.* (1997 and 2001). Amplification products were analysed by gel electrophoresis on 1% agarose gel, stained with ethidium bromide and visualised with UV exposure. Photographs taken were captured by BioCapture software (Vilber-Lourmat, Marne-la-Vallée, France).

Statistical Analysis

Differences in frequency of strains according to colony size, biovar and serogroup and its association with observed sanitary state were evaluated by GLM log-linear procedure of GenStat 8th edition software (VSN International Ltd., Hertfordshire, UK). Captured images were analysed by Bio-1D (Vilber–Lourmat) software. Similarity matrix was calculated with DICE and dendrogram was constructed by UPGMA procedure.

RESULTS AND DISCUSSION

All isolates were Gram negative rods that were indol, oxidase and catalase positive and failed to grow on MacConkey agar. The *P. multocida* specific PCR primers amplified the expected 460 bp fragment in all case. Colony morfology showed high variance of size and type (Figure 1). Diameter of the large colonies which represented the two third of the isolates was 1.5 to 2 mm. The small colonies were 0.5 to 1.5 mm and one isolate formed punctiform colonies. Colony type of two isolates was rough, and the

remaining divided between mucoid (n=13) and smooth (n=17). Most often detected capsule biosynthesis gene was *capA* (53%) and *capF* (28%). Only three (9%) *capD* isolates were found, and further three did not possess any of the *cap* genes known recently. Differences according to the health state of the source rabbits were not detected in contrary to the earlier findings of Rideaud and Coudert (1994) that isolates developing large and mucoid colonies were more pathogenic in rabbit. *P. multocida* strains isolated from rabbits in previous studies had mostly *capA* gene or belonged to capsular serogroup A. Serogroup D was found just in few cases by Ewers *et al*. (2006); Chengappa *et al*. (1982), Lu *et al.* (1983), although Percy *et al*. (1984) typed 33% of the strains into serogroup D. Some isolates did not possess any of the so far known capsule biosynthesis genes (Ewers *et al.*, 2006). *CapF* type rabbit isolates were first described by Jaglic *et al.* (2004) and in an another study (Jaglic *et al*., 2005) they found similar distribution of *A, D*, and *F cap* types within strains isolated from rabbits like in us. In the study of Digiacomo *et al.* (1991) the isolates from the same farm did not show comparable size of variability, the sampling was performed once and the number of samples was less.

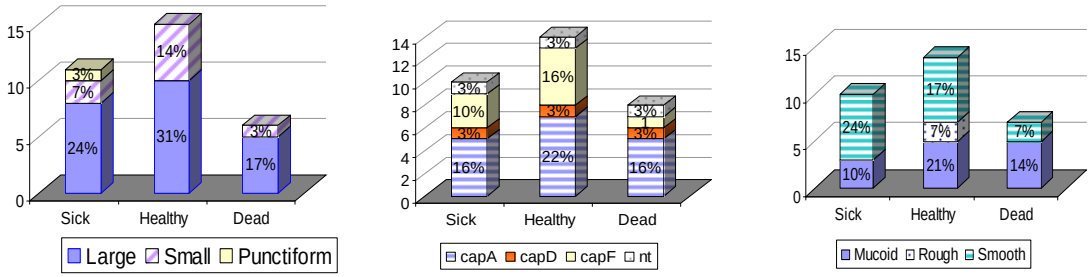

Figure 1: Frequency and distribution of the capsular types, colony size and colony morphology according to the health of the sampled rabbit

More than 70% of the isolates utilised galactose, glucose, fructose, mannose, mannitol and saccaharose, what are the key charesteristics of *P. multocida*. More than 80% were not fermenting sorbose and lactose. Maltose however was fermented by all but one. This is notable because non fermentation of maltose was also considered as key character in the identification of *P. multocida*. Similar results have already been published by Di Giacomo *et al.* (1991) who found high rate of strains fermenting maltose originated from NZW rabbits in one laboratory rabbit colony. Considering their ability to metabolize sorbitol, xylose, trehalose, arabinose and dulcitol the strains were typed according to Fegan *et al.* (1995) as biovar 1 (n=8), 3 (n=2), 6 (n=14) and 9 (n=1). Biovar 1, 3 and 9 have been assigned as *P. multocida multocida*, biovar 6 could not be allocated to any of the recognised subspecies of *P. multocida* (Figure 2). Further 7 isolates were not typed by the respective system. The biovar types were almost equally divided according to the health of the source rabbit, the small differences were not statistically proven. Typing into biovars according to Fegan *at al.* (1995) has not been attempted earlier from rabbit strains.

Cluster analysis based on the substrate fermentation separated seven clusters (Figure 2), which further discriminated the strains within capsule biosynthetic gene types and within same biovar. Three clusters containing strain 2, 5, 8, 32, 15 (Cl I) or single strains 3 (Cl VII) and 1 (Cl II) represent only isolates collected from sick or dead rabbits. Five strains in Cl I were unique in their capacity for metabolization of lactose, melezitose and turanose substrates. In the clusters remaining the proportion of the isolates originating from clinically healthy or from sick and dead rabbits were not very much different from 1/1.

Rep-PCR (Figure 3) also showed high genotypic variability of the strains. While 44% of strains in this selection came from clinically healthy animals, three clusters including strain 2, 3, 4, 5, 13, 24, 28 and 7, 8, 11, 25, 29 or the single strain 19 contains only 30, 20 and 0% of those, respectively. Another three clusters with strains 6, 9, 16, 17, 18, 20, 26, 27 and single strain 23 or strain 19 are represented by healthy isolates in 75, 100 and 100%. Although this is not a strict separation of strains by the sanitary state of the rabbit, some association between REP type and those could be supposed.

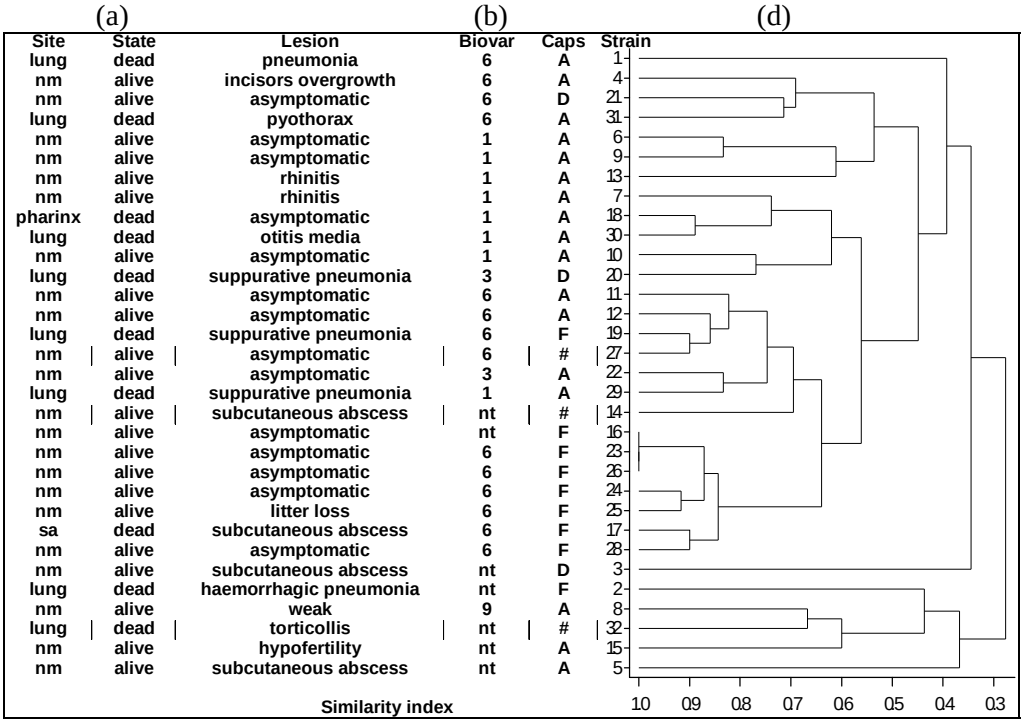

Figure 2: Sampling conditions (a), biovar and capsular genotype (b) of 29 *P. multocida* strains. Dendrogram (d) based on similarity matrix calculated from carbohydrate fermentation results

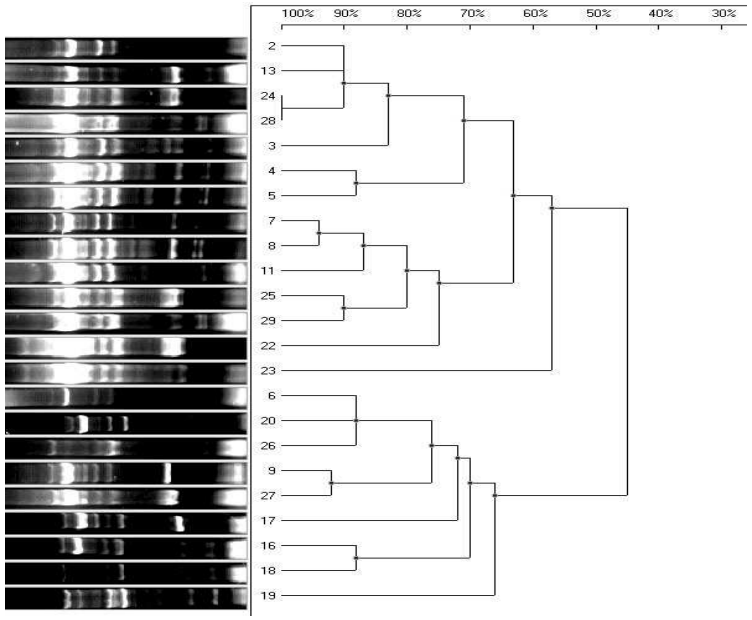

Figure 3: Electrophoresis pattern of REP-PCR product and dendrogram of genetic relatedness generated by REP-PCR of *P. multocida* DNA templates

CONCLUSIONS

High phenotypic and genotypic diversity were characteristic to *P. multocida* isolates collected from the same herd during time. Evaluation of fermentation results for 50 carbohydrates could further discriminate the strains within same biovar or subspecies. Clustering based upon phenotypic or genotypic data detected more groups of isolates separated above 40% similarity level. Strict association between sampling conditions and pheno- or genotypic groupings could not be detected.

ACKNOWLEDGEMENTS

This study was supported by the grant 46025/2004 from the Hungarian Ministry of Agriculture and Rural Development

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