THEORETICAL AND PRACTICAL ASPECTS OF THE DIFFERENTIATION OF *PASTEURELLA MULTOCIDA* STRAINS THROUGH THE STUDY OF THE BIOCHEMICAL KINETICS

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INTRODUCTION

Pasteurellosis is the most important cause of respiratory pathology in rabbits (PEETERS, 1995), with a prevalence of rhinitis in Spain near the 30% (ROSELL *et al.*, 1996), and it is also implicated in other pathologies (COUDERT *et al.*, 1986).

In the past, the characterization of bacterial strains was conducted by qualitative biochemical studies (DIGIACOMO et *al.*, 1991; BADIOLA *et al.*, 1992), morphological characteristics (RIDEAUD and COUDERT, 1994) and serological typing (HEDDLESTON *et al.*, 1972).

Within the last years, first with financing of the INIA (INIA-9071) and thereinafter with financing of the own sector, the IRTA has developed a technique that allows to find out similarities and differences between bacterial strains, and that has demonstrated to be very effective in epizootiological studies as well as in prophylactic vaccination studies.

Though the study discussed below is focused, fundamentally, in strains of *Pasteurella multocida* isolated from rabbits, the same kind of study has proven to be equally satisfactory for strains of *P.multocida* from other origins than rabbits, and also for some other species of microorganisms, such as *Pasteurella haemolytica*, *Salmonella* spp., *Escherichia coli* and *Staphylococcus* spp. isolated from different origins.

In this paper we will describe de principles of the method, and some practical aspect that we have studied in the last two years.

PRINCIPLES OF THE TECHNIQUE

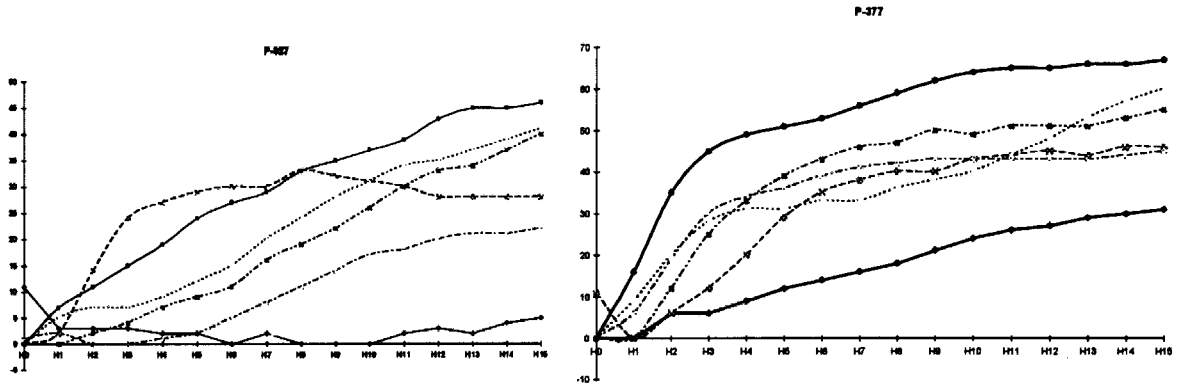
The automated system (Vitek, BioMJrieux) used by our group for bacterial identification, makes use of the biochemical activities of the microorganisms to classify them into bacterial species, and gives an information that, till now, nobody has been using.

This information represents the quantification of the changes that bacteria are able to produce in different culture media, upon possessing, or not, some active genes. Furthermore, as the system performs automatic readings each hour, the changes are not only end point readings. Figure 1 shows the graphic representation of some biochemical tests of two different *P.multocida* strains isolated from rabbits. The originality of the method proposed by our group, consists in the exploitation of the information outputted by de Vitek System, to compare the biochemical kinetics between several bacterial strains, and to evaluate their degree of similarity.

Starting with the hypothesis of the fact that the differences between strains would be real - not due to the technique itself - and of the fact that these differences must be due to changes in the structure/function of the proteins involved in the bacterial metabolism, and by consequence, to changes in the genoma, such differences would be a measure of the degree of genetic homology (an important data for epidemiological studies) and of the degree of antigenic homology between the analyzed microorganisms (an important data from the

vaccination point of view, if part of the differences are the reflections of the variations in the surface proteins or in the proteins involved in the synthesis of the components of the surface).

Figure 1: Biochemical kinetics of two strains of *P.multocida*



Data processed to compare the bacterial strains, consist of each one of the differences between all the hours (15), for each one of the biochemical characteristic studied (22).

Using a computer program designed by the IRTA itself, all the information provided by the system (330 data for each strain) is summarized in a graph of distances, which allows us to group the different bacterial strains by their degree of homology. This system has the advantage, over the molecular biology techniques that probably will take us to the same conclusions (ZHAO *et al.*, 1992), of allowing us to compare, with considerable facility, a great number of strains at the same time.

To reduce the important effect related to the adaptation of microorganisms to the *in vitro* growth, all bacterial strains studied were serially subcultured five times before the biochemical study was initiated.

PRACTICAL APPLICATIONS

We will describe some examples of the application of the technique designed by the IRTA; a summary of them follows:

1) Epidemiological studies:
   1.a.) General epidemiology in an specific geographic area.
   1.b.) Study of the degree of relationship between strains isolated from a rabbitry, and the farms which purchase animals from it.

2) Studies on the differences between strains isolated from rabbitries to:
   2.a.) Determine the similarities/differences between strains isolated from animals within a particular farm.
   2.b.) Select all the different strains isolated from a rabbitry to produce autobacterins.

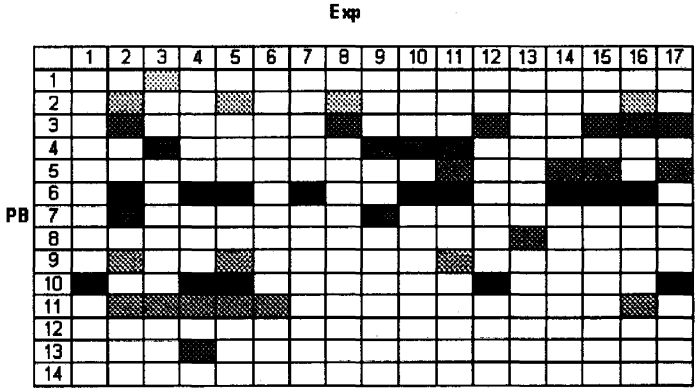
3) Pursue a special *Pasteurella* strain, inoculated in to a rabbit, in:
   3.a.) Challenge studies.
   3.b.) Studies to select bacterial strains with a low degree of virulence.

1) Epidemiological studies:

1.a.) General epidemiology in a specific geographic area

In Table 1, we could see the relations between rabbitries and *P. multocida* strains isolated from 17 farms (Exp), in a specific area of Spain (Navarra) during 1994. In this study, we isolated more than 100 strains of *Pasteurella* which could be grouped in 14 different clusters by the biochemical kinetic study (PB: Biochemical Profile). The degree of relationship between rabbitries, according to bacterial strain similarities, is very high, with PB-6 and PB-14 present in 9 different farms, and other 7 different PB's isolated from more than two different rabbitries. As can be seen in this table, more than one PB is present in all the 17 rabbitries. In this study we were able to find out some kind of relationship between the same PB and the epidemiological interrelations between farms.

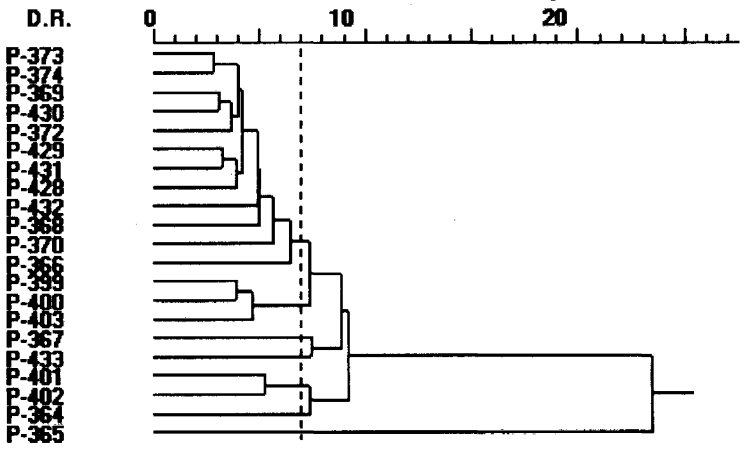
Table 1: Relationship between isolates of *P. multocida* in 17 rabbitries located in the area of Navarra.



1.b.) Study of the degree of relationship between strains isolated from a rabbitry, and the farms which purchase animals from it

With our methodology, we can the study of the degree of association amongst strains isolated in two different farms. Figure 2 shows the similarities between strains isolated in a rabbitry which produces genetically selected breeders (strains P-428 to P-433), and two farms which receive animals from that rabbitry (P-364 to P-374 and P-399 to P-403). In this figure, all the strains which form a cluster at a relative distance (D.R.) less than or equal to 7 units, are considered to be identical strains.

Figure 2: Dendogram of strains isolated from tree rabbitries commercially related.



2) Studies on the differences between strains isolated from rabbitries to :

2.a.) Determine the similarities/differences between strains isolated from animals within a particular farm

In Figure 2 the important degree of similarity amongst isolates from different origins is represented. It is very frequent, almost in all rabbitries, the isolation of identical strains from nose, lung and other pathologies. We isolated very similar strains from associated pathologies, such as those obtained from the enteric (peritonitis) and pulmonary (pleuritis) mucosa.

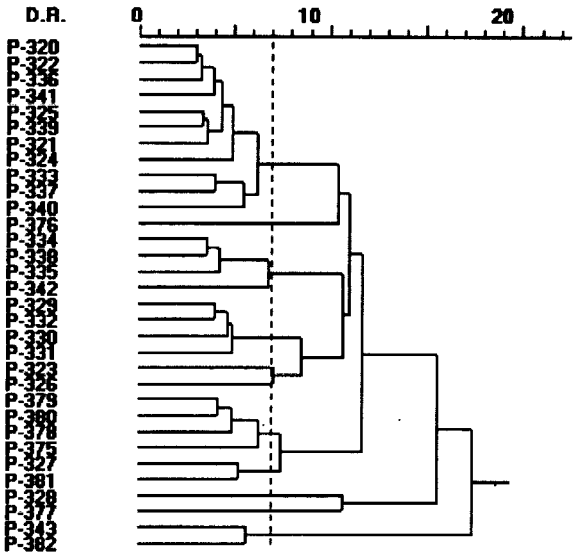
In other cases, in addition to the isolation of identical strains in different animals, it was possible to isolate, as well, different bacteria in the same animal. In some examples, the degree of dissimilarity between those strains was greater than 15 or 20 D.R. units.

2.b.) Select all the different strains isolated from a rabbitry to produce autobacterins

Figure 3 shows the dendogram representing the bacterial isolates from a rabbitry in two different periods of time (P-320 to P-343 and P-375 to P-382), with the introduction of a complex autobacterin between the two periods. In this figure, it could be seen that, always, all bacterial strains isolated in the first period, were not isolated any more in the second one. This finding could be explained by the application of an autobacterin, produced with a group of strains representing all the different clusters initially present in the rabbitry.

In the second analysis strains included in different clusters appeared, and only two strains identical to strains isolated during the first study could be isolated again.

Figure 3: Dendogram of bacterial strains isolated from a rabbitry at two different periods



3) Pursue a special *Pasteurella* strain, inoculated in to a rabbit, in:

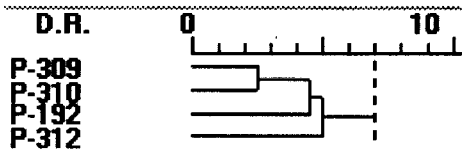
3.a.) Challenge studies

The study of the biochemical kinetics, allows the differentiation of a particular bacteria, once inoculated into animals and reisolated from them, between other *Pasteurella* strains isolated at the same time.

Figure 4 illustrates the case mentioned above with a challenge strain (P-192, intratacheally inoculated) that was identical to a series of strains isolated from the pneumonic lungs of the animals challenged.

This kind of findings have been also confirmed in other types of challenges and by different routes of inoculation.

Figure 4: Strains related n a bacterial challenge



3.b.) Studies to select bacterial strains with a low degree of virulence.

In a study planed to obtain an attenuated strain of *P.multocida* that will be used in vaccinial prophylaxis, and which is still in development, it has been possible to obtain and identify, after animal inoculation, related bacterial strains with an incomplete attenuation.

The study of the biochemical kinetics is very useful in the elimination of strains with any degree of residual virulence, and in the selection of strains with the lowest degree of virulence or without any pathological manifestations.

The method could be employed to pursue a live vaccinial strain, in cases of industrial plagiarism, allowing for the defence and validation of the product.

Besides all the usages mentioned above, the analysis of the biochemical kinetics can detect the changes related with bacterial attenuation, or with the adaptation to *in vitro* growth, and probably will predict the degree of reduction in virulence.

Figure 5 shows the relationship between a field isolate of a bacterial strain with low virulence (P-108/91), four sub-strains derived from it (P-10891***), after a series of 60 *in vitro* subcultures, and some strains (P-410 to P-414) isolated from animals injected with two of the sub-strains (incomplete attenuation).

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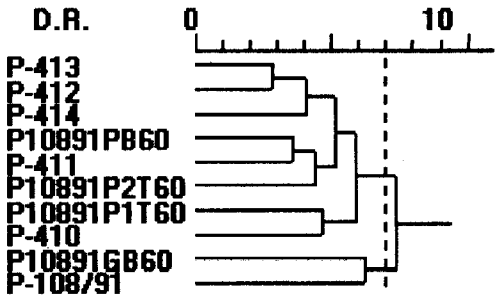
Figure 5: Relationship between sub-strains of *P.multocida*, the original strain and some field isolates from complicated cases.



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