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# BIOLOGICAL FEATURES OF *Pasteurella multocida* STRAINS ISOLATED FROM RABBITS IN THE NORTHEAST AREA OF SPAIN.

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## ABSTRACT

In the present study, 51 industrial type rabbit-raising farms, with a mean of 250 does within each farm, have been investigated to isolate a representative sample of *Pasteurella multocida* strains that can be found in the northeast part of Spain.
During a period of time of about 2 years, we have found 41 farms that were positive, and from which we were able to isolate 108 strains of *P.multocida*.
One hundred of those strains have been further analyzed, to establish their biochemical patterns in 50 different biochemical tests, to evaluate its susceptibility to 16 different antibiotics and to assess its capsular and somatic antigenic composition.
We have found very interesting relationships between some of the biological features of *P.multocida* strains, as well as between them and the body site of origin of the isolate.

## INTRODUCTION

From previous epidemiological field studies (Rosell et al., 1982), it can be assumed that with a correct management of the animals, and with the use of a specific medication, the rate of respiratory problems can be lowered to figures near a 15%. To improve these levels, three years ago we decided to undertake a more complex study, involving the foremost microorganism incriminated in those problems (Morisse, 1979), as well as in cutaneous processes (Hervouet and Nouaille, 1985), auricular lesions (Coudert et al., 1986) and reproductive disorders (Holmes et al., 1983): *Pasteurella multocida*.
In this paper, we are describing the results concerning to the body site of origin of the isolate, to their biochemical and antigenical properties, and the results of the antibiotic susceptibility tests, for the 100 strains of *Pasteurella multocida* isolated from the animals of 41 rabbit-raising farms located in the northeast zone of Spain.

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## MATERIALS AND METHODS

- *Farms*: During the period 1989-1991, 51 industrial type rabbitries were analyzed (with a mean number of 250 does by farm).
A farm was not analyzed any more once it gave a positive isolation of *Pasteurella* or after 25 samples have been analyzed (5 does, 9 nasal swabs and 11 animals, culled for several reasons).

- *Bacterial isolation*: The method of isolation chosen was direct plating on Blood Agar Base n°2 (Oxoid)[a], with a 6% of Horse Blood Defibrinated (Oxoid). We have not used mouse inoculation method, because of the possibility to increase the recovery of D-type strains (Cowart and Backström, 1984).
After 24 hours of cultivation at 37°C, the colonies visually resembling those of *Pasteurella* were selected and subcultivated again on to a fresh Blood Agar plate.

- *Biochemical characterization*: Those microorganisms suspected of being *Pasteurella multocida*, once purified, were inoculated on to GNI and GPI cards of the VITEK-AMS System[b], to proceed to its biochemical identification and metabolic study. The unique features of this system, made possible the accurate following of the growth rate, and of the arising or disappearing of certain metabolites, by means of hourly readings and during a period of time of 15 hours of cultivation, with a total of 48 different biochemical tests and two growth controls.
At the same time, we made a Gram Stain of the isolate, and the Catalase and Oxidase tests.

- *Antigenic characterization*: For capsular typing, we used the techniques described by Carter and Subronto (1973) and Carter and Rundell (1975). For the typing of A-type strains, we used a strain of *Staphylococcus aureus* kindly supplied by Dr. J.Peeters.
For the study of somatic antigens, we used reference antisera supplied by the National Veterinary Services Laboratory, Ames (Iowa, USA), and the technique described by Heddleston et al. (1972). Once extracted, the LPS of each strain was kept at -70°C until the moment of being challenged with the reference antisera.

- *Antimicrobial susceptibility studies*: To evaluate the antibiotic susceptibility of the strains isolated, two different kinds of studies were carried out.
In the first one, we were using the VITEK-AMS System and inoculating the GNS-UA cards. By this method we were able to assess the growth of the *Pasteurella* strains in the presence of different concentrations of Ampicillin, Cefazolin, Cefoxitin, Ceftriaxone, Gentamicin, Nitrofurantoin, Norfloxacin, Tetracycline, Ticarcillin, Tobramycin and Trimeth-Sulfa.
In the second one, we made agar plate susceptibility tests using Mueller-Hinton Agar (Difco)[c] supplemented with 3% Horse Blood Defibrinated (Oxoid), according to the method described by Bauer et al. (1966), and the Neo-Sensitabs tablet system[d], employing the following antibiotics:

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[a] Unipath Limited, Basingstoke, Hampshire, England.

[b] Vitek Systems, bioMérieux, USA.

[c] Difco Laboratories, Detroit, Michigan, USA.

[d] A/S Rosco, Denmark.

Chloramphenicol, Sulphonamides, Penicillin High, Tetracycline, Spiramycin, Gentamicin and Streptomycin.
In the GNI and GPI Vitek cards, the growth of the microorganisms in the presence of the antibiotics Polymyxin B, Novobiocin, Bacitracin and Optochin is also analyzed.

- *Statistical analysis*: To study the similarities/differences between strains, a discriminant analysis was used and estimated with the help of the SAS-STAT Software[a].
In the studies of association between different characteristics, 2-by-2 contingency tables were used, in addition to an association analysis based on the chi-square statistic ($\chi^2$). When considering antibiotic susceptibility tests, microorganisms were sorted in to two categories: more susceptible or less susceptible than the mean of the inhibition zones for the 100 strains analyzed. Moreover, for the statistical analysis of the possible relationships between the sizes of the inhibition zones for the different antibiotics considered, a transformation in to quarter sections of the sizes was applied. Quarters were delimitated by means of three values, calculated from the mean and the standard deviation of the inhibition zones of the different antimicrobials: the first value (point 1), was the result of subtracting the product of the standard deviation by the constant 0.6745, from the mean; the second value (point 2), was the mean; and the third value (point 3), was calculated by adding to the mean the product of 0.6745 by the standard deviation. Quarters were defined as follows: Quarter 0, that one which contains values lesser than point 1; Quarter 1, that one which comprises values between points 1 and 2; Quarter 3, that one which comprises values between points 2 and 3; and Quarter 4, the one with values greater than point 3.

## RESULTS AND DISCUSSION

Until now, from the 51 rabbitries studied, 41 have been found positive (80.39%), and from them 108 strains of *Pasteurella multocida* have been isolated.
Of those 108 strains, 100 have been studied further, as described in Materials and Methods, and have been preserved in skim milk at -70°C. The remaining 8 strains, after its identification as *Pasteurella*, were lost, for several reasons, in different stages of the study.
From the 108 *P.multocida* isolated, 50 were obtained from nostrils (mostly from animals with signs of rhinitis or from animals with other concurrent processes from which *P.multocida* was isolated too), 20 strains were isolated from auricular necrosis lesions, 12 from cutaneous abscesses, 13 from lungs, 11 from pyometra and 2 from peritonitis.
From that stand point, all the data and results exposed are referred to the 100 bacteria fully studied.
In **Table 1** are sown the results, in percentages of positivity, for some of the biochemical characters studied, for all the strains and referred to the body site of origin.
Our results are slightly different from those published by some other authors (Lu et al., 1978; Heddleston, 1976; Spanoghe, 1984), especially as regards as the results of fermentation of

[a] SAS Institute Inc., Cary, NC, USA.

Xylose, Sorbitol, Trehalose, Arabinose, Raffinose, Maltose, Salicin and Ornithine Decarboxylation.

| | GLU | XYL | SOR | ORN | BAC | OPT | TZR | NOV | DEX | RIB | SUC | LAC | ARA | MAN | TRE | OXI | CAT |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N | 59 | 43 | 63 | 74 | 92 | 100 | 24 | 35 | 50 | 91 | 100 | 0 | 0 | 100 | 4.3 | 100 | 100 |
| L | 69 | 54 | 55 | 92 | 89 | 100 | 23 | 38 | 31 | 85 | 100 | 0 | 0 | 100 | 23 | 100 | 100 |
| E | 67 | 22 | 44 | 94 | 93 | 93 | 22 | 11 | 28 | 72 | 100 | 0 | 0 | 100 | 11 | 100 | 100 |
| S | 55 | 45 | 45 | 73 | 100 | 100 | 9 | 36 | 45 | 82 | 100 | 0 | 0 | 100 | 0 | 100 | 100 |
| P | 50 | 0 | 50 | 100 | 0 | 100 | 50 | 0 | 100 | 100 | 100 | 0 | 0 | 100 | 0 | 100 | 100 |
| U | 10 | 50 | 80 | 50 | 100 | 100 | 10 | 40 | 50 | 100 | 100 | 0 | 0 | 90 | 0 | 100 | 100 |
| MEAN | 56 | 41 | 58 | 78 | 91 | 100 | 21 | 31 | 44 | 87 | 100 | 0 | 0 | 99 | 7 | 100 | 100 |

Table 1.- Results of the biochemical tests and body sites of origin for the 100 strains of *Pasteurella multocida*.
N = nostrils, L = lungs, E = ears, C = cutaneous, P = peritonitis, U = uterus.

This might be due to a side effect of the method used for the study, or may indicate that the strains isolated are biotipically different.
The results obtained for Glucose oxidation, fermentation of Xylose, Sorbitol and Dextrose, and the growth in 3.75 $\mu$g/ml Novobiocin, lead to the differentiation of 16 biotypes amongst the 100 strains isolated. In Table 2 the differential characters of each biotype, the incidence of strains and the distribution by body site of origin are shown. The most frequently isolated biotypes were 7, 9 and 10.

| | GLU | SOR | XYL | NOV | DEX | TOTAL | N | L | E | S | P | U |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Biotype 1 | - | - | - | - | - | 5 | 2 | 0 | 3 | 0 | 0 | 0 |
| Biotype 2 | - | - | - | - | + | 9 | 3 | 1 | 1 | 2 | 0 | 2 |
| Biotype 3 | - | + | - | - | - | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| Biotype 4 | - | + | - | - | + | 8 | 3 | 0 | 1 | 0 | 1 | 3 |
| Biotype 5 | - | + | - | + | + | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| Biotype 6 | - | + | + | - | - | 5 | 1 | 2 | 1 | 1 | 0 | 0 |
| Biotype 7 | - | + | + | + | - | 14 | 7 | 1 | 1 | 1 | 0 | 4 |
| Biotype 8 | - | + | + | + | + | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| Biotype 9 | + | - | - | - | - | 14 | 3 | 4 | 5 | 2 | 0 | 0 |
| Biotype 10 | + | - | - | - | + | 14 | 9 | 1 | 1 | 2 | 1 | 0 |
| Biotype 11 | + | + | - | - | - | 5 | 3 | 0 | 2 | 0 | 0 | 0 |
| Biotype 12 | + | + | - | - | + | 2 | 1 | 0 | 1 | 0 | 0 | 0 |
| Biotype 13 | + | + | + | - | - | 5 | 3 | 0 | 1 | 0 | 0 | 1 |
| Biotype 14 | + | + | + | - | + | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| Biotype 15 | + | + | + | + | - | 7 | 3 | 2 | 1 | 1 | 0 | 0 |
| Biotype 16 | + | + | + | + | + | 8 | 4 | 2 | 1 | 1 | 0 | 0 |

Table 2.- *Pasteurella* biotypes and number of strains related to the body site of origin.
N = nostrils, L = lungs, E = ears, C = cutaneous, P = peritonitis, U = uterus.

Table 3 shows the results of the somatic antigen studies. The serogroups found and the percentage of strains within each serotype are presented.

These results agree, as regards as somatic serotypes found, with data published by Spanoghe (1984) and Okerman and Devriese (1986) in Belgium, by Morisse (1979) and Laval (1989) in France, and by Chengappa et al. (1982) in the USA. But they do not agree in the percentages of strains found within each serotype.

All data collected, in addition to the lack of concordance with data of some other countries, might be an indication of a typical and characteristic strain distribution pattern within each country, or region inside a country, making thus impossible the extrapolation of results from a country to another.

| SOMATIC TYPE | FREQUENCY OF ISOLATES |
|---|---|
| 1 | 4 |
| 3-A | 16 |
| 3-B | 60 |
| 3-AB | 7 |
| 12 | 4 |
| 12,7 | 2 |
| 12,7,4 | 6 |
| Untypeable | 1 |

Table 3.- Somatic types of *Pasteurella multocida* isolated from rabbits.

It is important to remark the fact that not all serotypes are absolutely identical, because, as can be seen from Figure 1, some bacteria considered as being type 3 give also precipitation bands of "non antigenic identity". The same fact has been observed for strains of serotype 12 too. Now, we are studying further that phenomenon in all the other strains of *Pasteurella*, and also which antigens can be involved in it.

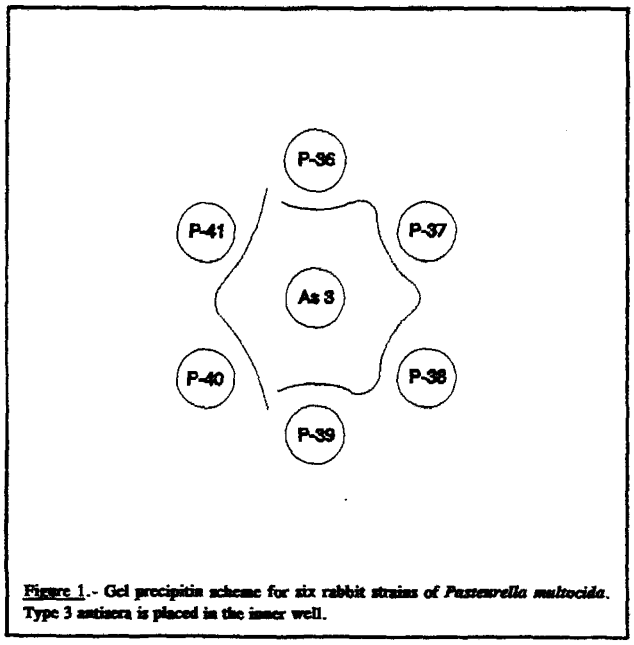


Figure 1.- Gel precipitin scheme for six rabbit strains of *Pasteurella multocida*. Type 3 antisera is placed in the inner well.

When considering capsular typification, most of the *Pasteurella* strains isolated were A-type (75%), whilst only a 10% were D-type. The remaining 15%, could not be definitely typified. All these data fully agree with those already published by some other authors (Chengappa et al., 1982; Laval, 1982; Lu et al., 1978; and Spanoghe, 1984).

The results obtained for the susceptibility tests carried out in the AMS-Vitek System, are shown in Table 4. They are in accordance with data published by Maire (1989), except in the point that this author reports the finding of Trimeth-Sulfa resistant strains and we have not found any. Table 5 illustrates the results for the percentages of the strains classed as sensitive/intermediate/resistant, related to the body site of origin, with Neo-sensitabs tablets. From that table, some interesting conclusions can be outlined: a) The only Penicillin resistant strains, were isolated from lungs; b) Gentamicin resistant strains, were obtained from nostrils and lungs of some animals of the same rabbitrie, and from nostrils of 1 animal in another farm; c) The percentage of strains Streptomycin resistant is slightly higher in the

| ANTIMICROBIAL | S | I | R |
|---|---|---|---|
| Ampicillin | 100[a] | 0 | 0 |
| Cefazolin | 100 | 0 | 0 |
| Cefoxitin | 100 | 0 | 0 |
| Ceftriaxone | 100 | 0 | 0 |
| Gentamicin | 82 | 12 | 6 |
| Nitrofurantoin | 100 | 0 | 0 |
| Norfloxacin | 100 | 0 | 0 |
| Tetracycline | 98 | 2 | 0 |
| Ticarcillin | 100 | 0 | 0 |
| Tobramycin | 88 | 10 | 2 |
| Trimeth-Sulfa | 100 | 0 | 0 |

Table 4.- Antimicrobial susceptibility test using the AMS-Vitek System.
[a] Frequency of isolates.

isolates coming from auricular necrosis problems; d) Strains isolated from lungs were, in general, more resistant than strains from other origins.

All those findings might indicate a possible relationships between antibiotic susceptibility and degree of virulence, as has already been pointed out by some other authors (Lee et al., 1988; Hancock, 1984).

| | NOSE | LUNG | EAR | SKIN | UTERUS | TOTAL |
|---|---|---|---|---|---|---|
| Chloramphenicol | 100/0/0 [a] | 100/0/0 | 100/0/0 | 100/0/0 | 100/0/0 | 100/0/0 |
| Sulphonamides | 48/15/37 | 38/8/54 | 56/0/44 | 36/9/55 | 30/30/40 | 46/2/42 |
| Penicillin | 98/2/0 | 77/8/15 | 100/0/0 | 100/0/0 | 100/0/0 | 96/2/2 |
| Tetracycline | 100/0/0 | 100/0/0 | 100/0/0 | 100/0/0 | 100/0/0 | 100/0/0 |
| Spiramycin | 65/22/13 | 47/15/38 | 66/17/17 | 55/27/18 | 30/50/20 | 58/24/18 |
| Gentamicin | 94/2/4 | 62/23/15 | 72/28/0 | 100/0/0 | 90/10/0 | 86/10/4 |
| Streptomycin | 61/26/13 | 15/77/8 | 50/22/28 | 46/36/18 | 40/50/10 | 48/37/15 |

Table 5.- Percentage of different susceptibility categories referred to the body site of isolation.
[a] Percentages of Sensitive/Intermediate/Resistant.

The analysis of the inhibition zones expressed as quarter sections, shows a very close relationship between the different antimicrobials assayed for the same strain, because a 47% of the strains have 5 or more antibiotics located at the same quarter, and a 97% of the strains have 5 or more antibiotics located at the same quarter and in the immediate upper one, or at the same quarter and in the immediate lower one. This phenomenon, in addition to other interesting relationships, is presented in the array of results of the association analysis based on the chi-square statistic ($\chi^2$) showed in Table 6 and Figure 2 and Figure 3. Statistically significant relationships have been observed between Sorbitol and Xylose, and between the susceptibility to Novobiocin and the fermentation of these carbohydrates (all bacteria Novobiocin resistant ferment Sorbitol and Xylose, except one strain unable to ferment Xylose). Finally, we would like to remark the relationship between the ability to oxidize Glucose and the degree of susceptibility to the antimicrobials assayed, meaning a greater resistance in the strains which cannot oxidize Glucose than in the strains that are able to do so.

| | GLU | XYL | SOR | ORN | BAC | OPT | TZR | NOV | DEX | RIB | CHL | SUL | PEN | TET | SPI | GEN | STR |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GLU | - | 0.6 | 3.3 | 6.6 | 2.0 | 1.2 | 9.5 | 1.0 | 0.0 | 2.6 | 6.9 | 2.9 | 4.8 | 0.0 | 8.3 | 6.6 | 4.2 |
| XYL | NS | - | 50.3 | 11.7 | 0.8 | 3.7 | 7.2 | 57.8 | 10.9 | 0.0 | 1.6 | 3.9 | 2.5 | 2.2 | 3.2 | 0.2 | 0.0 |
| SOR | NS | *** | - | 6.6 | 2.3 | 5.0 | 0.8 | 32.5 | 3.4 | 2.2 | 1.0 | 5.3 | 1.1 | 2.4 | 8.4 | 1.1 | 0.0 |
| ORN | ** | *** | * | - | 4.8 | 4.7 | 2.4 | 7.3 | 5.2 | 1.8 | 0.7 | 0.3 | 4.2 | 0.1 | 4.9 | 0.1 | 0.1 |
| BAC | NS | NS | NS | * | - | 67.6 | 4.1 | 0.0 | 9.8 | 5.5 | 0.3 | 0.1 | 0.1 | 3.3 | 0.4 | 0.0 | 1.8 |
| OPT | NS | NS | * | * | *** | - | 3.9 | 0.2 | 15.4 | 6.4 | 0.8 | 0.4 | 0.4 | 0.5 | 0.5 | 0.0 | 0.6 |
| TRZ | ** | ** | NS | NS | * | * | - | 5.7 | 0.1 | 4.0 | 0.0 | 0.1 | 0.4 | 8.9 | 1.0 | 0.4 | 0.3 |
| NOV | NS | *** | *** | ** | NS | NS | * | - | 2.5 | 0.4 | 0.9 | 7.4 | 2.7 | 0.7 | 6.1 | 0.1 | 0.0 |
| DEX | NS | *** | NS | * | ** | *** | NS | NS | - | 8.0 | 0.3 | 0.8 | 0.0 | 0.6 | 0.7 | 1.9 | 0.2 |
| RIB | NS | NS | NS | NS | * | * | * | NS | ** | - | 4.7 | 0.4 | 6.8 | 5.0 | 7.6 | 10.0 | 8.0 |
| CHL | ** | NS | NS | NS | NS | NS | NS | NS | NS | * | - | 4.8 | 41.0 | 21.1 | 16.6 | 33.9 | 21.1 |
| SUL | NS | * | * | NS | NS | NS | NS | ** | NS | NS | * | - | 9.2 | 5.9 | 9.9 | 5.4 | 5.7 |
| PEN | * | NS | NS | * | NS | NS | NS | NS | NS | ** | *** | ** | - | 11.6 | 31.6 | 44.5 | 29.3 |
| TET | NS | NS | NS | NS | NS | NS | ** | NS | NS | * | *** | * | *** | - | 12.2 | 15.9 | 12.9 |
| SPI | ** | NS | ** | * | NS | NS | NS | * | NS | ** | *** | ** | *** | *** | - | 36.9 | 35.0 |
| GEN | ** | NS | NS | NS | NS | NS | NS | NS | NS | ** | *** | * | *** | *** | *** | - | 41.0 |
| STR | * | NS | NS | NS | NS | NS | NS | NS | NS | ** | *** | * | *** | *** | *** | *** | - |

Table 6.- Array for the association levels between biochemical characteristics and antimicrobial susceptibility. $\chi^2$ values in upper triangular half, and significant levels in lower triangular half. (NS = non significant, * = $p<0.05$, ** = $p<0.01$, and *** = $p<0.001$)

As final conclusions that can be drawn from the present study, we would like to remark the following points:

- *Pasteurella* strains can vary in their biochemical and antibiotic susceptibility patterns in a statistically related way.
- As regards as biotypes and percentages of serotypes, our findings are different from those of some other countries.
- Not all bacteria included in the same somatic type are antigenically identical.
- The homogeneity in the antibiotic susceptibility pattern could be an indication of the existence of variations in the outer membrane proteins.

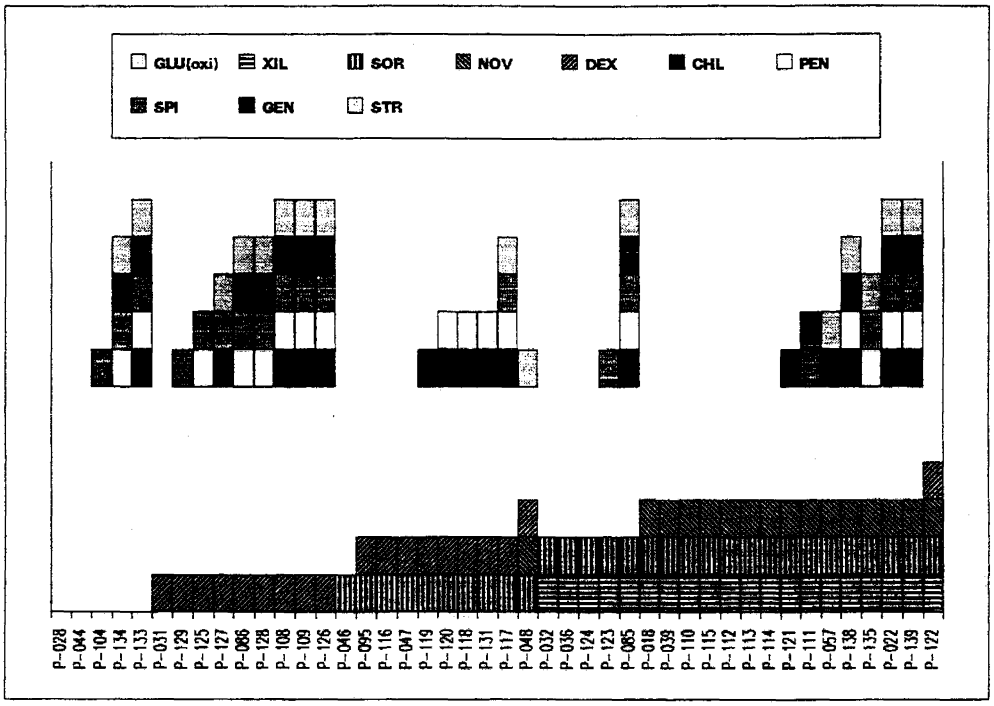


**Figure 2.- Relationship between biochemical and antimicrobial susceptibility patterns of non Glucose oxidative *Pasteurella multocida*.**

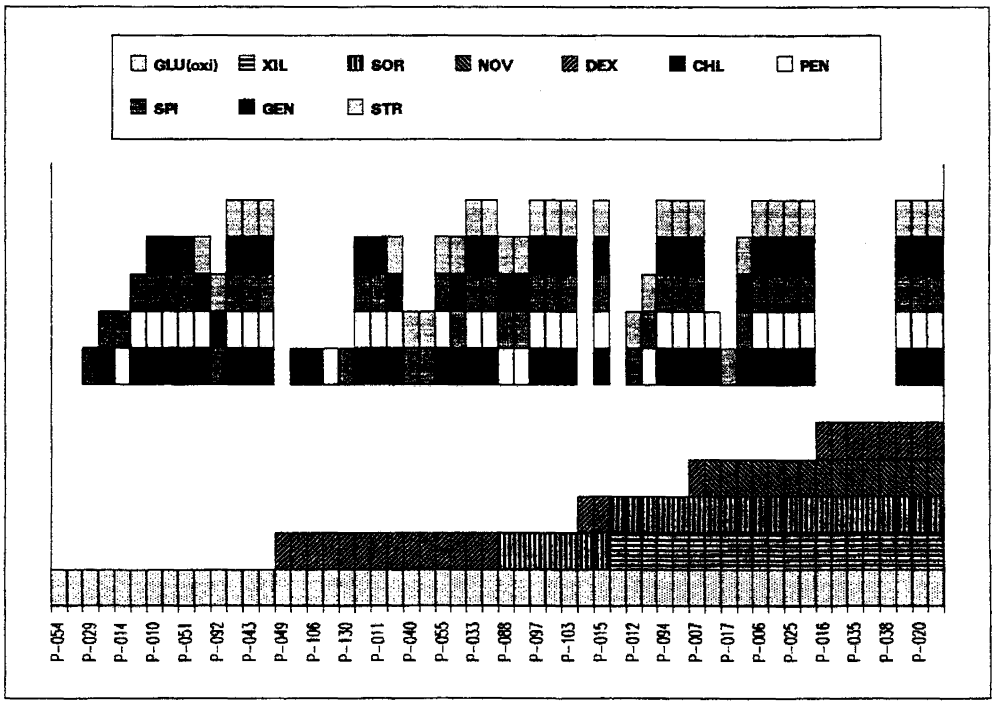


**Figure 3.- Relationship between biochemical and antimicrobial susceptibility patterns of Glucose oxidative *Pasteurella multocida*.**

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