KINETICS OF EXCRETION IN MILK OF SOME ANTIMASTITIS DRUGS

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Summary. - A study on the kinetics of excretion in milk of some antimastitis drugs is described. The five antibiotic molecules used were detected by confronting two methods: the Galesloot-Hassling method which, even though specific for the detection of penicillin in milk, is officially used for the detection of antibiotic residues in general; and the M. luteus paper disk assay, which uses M. luteus ATCC 9341 as test microorganism. The obtained results show that withdrawal times for the assayed molecules are longer than those declared and that M. luteus is more efficient for the detection of kanamycin residues.

Riassunto (Cinetica di eliminazione di alcuni farmaci antimastitici nel latte). - Si descrive uno studio sulla cinetica di eliminazione di alcuni farmaci antimastitici nel latte. Le cinque molecole antibiotiche utilizzate sono state ricercate impiegando a confronto i due metodi: il metodo Galesloot-Hassling specifico per la ricerca della penicillina nel latte, ma ufficialmente utilizzato per la ricerca dei residui antibiotici in generale; e il metodo M. luteus paper disk assay che utilizza come germi di saggio il M. luteus ATCC 9341. Dai risultati si dimostra che i tempi di sospensione dichiarati sono inferiori a quanto riconosciuto e che il M. luteus risulta più efficace nell'evidenziare residui di kanamicina.

Introduction

Bovine mastitis is an inflammatory disease of the mammary gland which produces changes both in the gland itself and in the milk secreted. The most frequent disease in industrial breeding, it is also the cause of the heaviest losses for the dairy industry [1, 2].

Mastitis is the response to the imbalance between the natural defense mechanisms of the teat and the mammary gland, and the amount and pathogenicity of microorganisms in contact with the opening of the teat canal (mainly streptococci, staphylococci, coliforms) [3, 4].

There are various factors which influence the impact of etiologic agents. The type of livestock raising (grazing, stalls), its structure and the sanitary norms applied, including the cleaning and disinfection of milking machines, are reckoned as factors of primary importance [4-7].

At the beginning, a successful treatment was the sole application of penicillin and penicillin-like molecules. In Italy, the method of Galesloot-Hassling [8], extremely sensitive to penicillins, was officially used for the detection and measurement of residues in milk.

Later on, the appearance of penicillin-resistant strains and the increased incidence of etiologic agents other than streptococci required the application of different molecules. Due to their efficiency, the new formulations became of widespread use in almost no time. However, the method for the detection of residues in milk contemplated by the Italian legislation has not been adapted accordingly. This method is still deemed suitable due to its intrinsic sensitivity and simplicity.

At present, the treatment of mastitis is based on pharmaceutical preparations having one or more antibiotic molecules associated to slow or fast release vehicles. Animals are treated during the dry pause or the lactating period. Drugs are administered directly into the teat canal (intramammary infusion), parenterally or, more rarely, via uterus [9].

When the antibiotic treatment is performed during the lactating period, the milk produced contains residues of the active principle until the end of withdrawal time [10]. Treatment during the dry period, favoured in some particular forms of mastitis, obviously avoids residual contamination of milk [11].

Determining factors in the excretion of antibiotics in milk are the chemical and physical characteristics of both the antibiotic and the pharmaceutical vehicle [2, 12]. The latter is defined as the substance, or group of substances, used as a support for a certain active principle and which influences its efficiency and its absorbance and excretion properties [13].
Among the various preparations used, suspensions, emulsions and ointments are those which entail a slow absorbance in the mammary gland and a longer persistence in milk [14].

The presence of antibiotics in milk raises two fundamental problems:

a) a technological problem in the dairy industry due to the partial or complete inhibition of starter cultures (S. thermophilus, L. bulgaricus), highly sensitive to antibiotics [15, 16];

b) a public health problem where three main aspects may be distinguished: toxicological, microbiological and immunopathological. The toxicological aspects are related to the health hazard posed by the ingestion of antibiotic residues (accumulation in kidney and liver, etc.) [17]. The microbiological aspects refer to the alteration of the intestinal bacterial flora and the growth of antibiotic-resistant strains [1, 17-19]. The immunopathological aspects are linked to the allergic properties of penicillins [20, 21]. These properties hold also true for other antibiotics under certain conditions and, in particular, for their impurities.

Regarding public health consequences in relation to the presence of residues in foodstuffs, the health authorities of different countries have adopted varying criteria [22]. In Italy the zero-tolerance level is required. Law 283 of April 20, 1962, in its article 5 (a) bans the marketing of foodstuffs whose composition diverts from the natural one. The Ministerial Decree of March 31, 1965 and its successive modifications do not allow the use of antibiotics for food preservation.

The official method for the detection of antibiotic residues in milk currently adopted by the Italian legislation [8] is an agar diffusion test which uses B. stearothermophilus strain calidolactis as test microorganism. This method
Table 1. Sensitivity to various antibiotic molecules expressed as minimum detectable concentration (μg/ml)

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>B. stearothermophilus</th>
<th>M. luteus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>0.005</td>
<td>0.01</td>
</tr>
<tr>
<td>Sodium benzylpenicillin (IU/ml)</td>
<td>0.002</td>
<td>0.003</td>
</tr>
<tr>
<td>Sodium ampicillin</td>
<td>0.04</td>
<td>0.15</td>
</tr>
<tr>
<td>Sodium dicloxacillin</td>
<td>0.04</td>
<td>0.15</td>
</tr>
<tr>
<td>Sodium oxacillin</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>Chlorotetracycline</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>Erythromycin lactobionate</td>
<td>0.5</td>
<td>0.005</td>
</tr>
<tr>
<td>Macrolides</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>Erythromycin sulfate</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Erythromycin lactobionate</td>
<td>3.5</td>
<td>0.1</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>0.1</td>
<td>0.002</td>
</tr>
</tbody>
</table>

has been devised for the detection of penicillin and penicillin-like molecules in milk. Since this strain is highly sensitive to some antibiotic molecules other than penicillin [23], it is routinely adopted for the detection of inhibitors in milk.

Therefore, this method has been used to study and determine withdrawal times for all the antimastitis drugs authorized in Italy for the last 20 years. However, this strain is rarely sensitive to some other molecules, especially macrolides. On the other hand, the pharmaceutical vehicle used in some formulations may contain ingredients which increase the persistence of the active principle, consequently slowing the absorption rates.

These considerations pose two important questions: could there be another method by which the detection limit of some antibiotics may be improved and are withdrawal times longer than what presently claimed to be?

Thus, the aims of the present paper are: a) to devise a method, complementary to the official one, which may improve the detection limit of the active agents in some of the antimastitis drugs present on the Italian market; b) to determine accurate withdrawal times for these drugs in order to ensure compliance with the law.

Fig. 3 - Inhibition zones obtained on B. stearothermophilus and M. luteus from mastitic (a) and healthy (b) udders after infusion of cloxacin.
† Last treatment; ——— B. stearothermophilus; ——— M. luteus

Fig. 4 - Inhibition zones obtained on B. stearothermophilus and M. luteus from mastitic (a) and healthy (b) udders after infusion of procaine penicillin G-
† Last treatment; ——— B. stearothermophilus; ——— M. luteus
Table 2. - Antimastitis products assayed

<table>
<thead>
<tr>
<th>Molecule(s)</th>
<th>Declared vehicle</th>
<th>N of animals</th>
<th>Declared withdrawal time (days)</th>
<th>Last sample: after end of treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oracillin</td>
<td>vaselin + liquid paraffin</td>
<td>1</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Dicloxacillin</td>
<td>vaselin + paraffin</td>
<td>9</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>vaselin + liquid vaselin</td>
<td>6</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Procaine penicillin-kanamycin</td>
<td>vaselin + paraffin</td>
<td>10</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>Aminosidine</td>
<td>vaselin + paraffin</td>
<td>10</td>
<td>5</td>
<td>10</td>
</tr>
</tbody>
</table>

Materials and methods

Method setup

Test microorganisms

The minimum inhibitory concentrations (MIC) of number of antibiotics were confronted for two microorganisms: *B. stearothermophilus*, test microorganism in the Galesloot-Hassassay, and *M. luteus*. Table 1 reports the results for both strains. According to the literature (24, 25), the latter is more sensitive to macrolides, oligosaccharides and rifamycins.

The same antimastitis formulas were parallely tested by:
- the Galesloot-Hassassay. Test microorganisms: *stearothermophilus*, strain *calidolactis*, and
- the *M. luteus* paper disk assay. Test microorganism: *M. luteus* ATCC 9341.

Sample preparation

*Agar seeded with B. stearothermophilus.* - The culture medium (8), melted and cooled down to 50 °C, was inoculated with a *B. stearothermophilus* spore suspension so as to obtain a concentration of 10⁶ c.f.u./ml. The seeded agar thus prepared was distributed in Petri dishes and a perfectly uniform, 0.9 mm deep layer obtained. The dishes were kept in the refrigerator before use, but never for more than 6 days.

*Agar seeded with M. luteus.* - Standard agar II (Merck) at pH 8, melted and cooled down to 50 °C, was inoculated with *M. luteus* cultured in a Brain Heart infusion broth for 24 h at 37 °C, so as to obtain a concentration of 10⁶ c.f.u./ml.

Fig. 3 - Inhibition zones obtained on *B. stearothermophilus* and *M. luteus* from mastitis (a) and healthy (b) udder after infusion of antimicrobials.

† Last treatment; –– *B. stearothermophilus*; ––* M. luteus*.
of 1 U/ml. The seeded agar thus prepared was distributed in Petri dishes, and a perfectly uniform, 2 mm deep layer obtained. The dishes were kept in the refrigerator before use, but never for more than 6 days.

Assay procedure. - For each milk sample, two paper disks, 13 mm diameter, soaked with 100 μl milk, were placed on the surface of the dishes seeded with B. stearothermophilus and M. luteus, respectively. By the end of their respective incubation times, the inhibitory activity was evidenced by the presence of clear zones of inhibition around the disks, which were measured in millimeters.

Evaluation of withdrawal times for some antimastitis drugs

Molecules examined

The following molecules, singly or associated, present in the formulas used for the antimastitis treatment of the cows in our sample were examined (Table 2): oxacillin, dicloxacillin, cloxacillin, procaine penicillin-kanamycin and aminosidine.

In order to assess withdrawal times, each affected quarter was infused with the product under examination (Table 2). The animal sample consisted of 36 Frisian cows, affected by clinically and bacteriologically diagnosed mastitis.

As of the second lactation after the last treatment, a milk sample was extracted from every healthy and diseased quarter, stored in a sterile bottle and analyzed according to the above described methods.

Results

The kinetics of excretion of the five antimastitis drugs examined (Table 2) are presented in Figs 1 through 5, which show that:

a) what could be expected from confronting MIC values is confirmed: B. stearothermophilus strain calidolactis, presents the highest sensitivity to penicillins. As for the association procaine penicillin-kanamycin, we underline that inhibition zones are attributable only to kanamycin, inactivation being carried out with penicillinase. In this case M. luteus is remarkably more sensitive to kanamycin than B. stearothermophilus is. The detection limit for this oligosaccharide is thus lowered. As far as aminosidine is concerned, our data - in agreement with those by other authors [26] - show that B. stearothermophilus is, again, more sensitive;

b) after treatment of affected quarters, molecules pass through to healthy quarters, in agreement with the data by other authors;

c) residues from the majority of the pharmaceutical products assayed persist in milk beyond their declared withdrawal times.

Discussion

A careful evaluation of the obtained results shows that the withdrawal times indicated on the products appear not to be accurate.

Therefore, a reexamination of the withdrawal times for the tested drugs would be advisable. More precise data will certainly help the user avoid the residue problem.

In the near future, it would also be advisable to determine the acceptable daily intake (ADI) for each molecule on the basis of toxicological studies. Such values could be the basis for the determination of maximum tolerance residue levels in milk - rather than absolute zero - which would allow a further analysis of withdrawal times. This criterion would facilitate the definition of the analytical methods to be applied for residue detection.

The obtained results confirm MIC data by which M. luteus is the best choice for the detection of kanamycin residues.

With respect to the passage of the active principle from affected to healthy quarters, the warning against using the milk from healthy quarters of cows under treatment for human consumption is reconfirmed.

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REFERENCES