Significance of the Presence of Bovine Milk β-Glucuronidase in Mastitis Detection

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ABSTRACT

The presence of β-glucuronidase enzyme in bovine milk was related both to the existence of major and minor pathogens and to somatic cell counts. The detection of this enzyme in whole milk was made possible by the use of p-nitrophenyl-β-glucuronide as a substrate. This detection allowed us to determine abnormal udder secretions with a high degree of specificity and sensitivity. The particular method of enzyme determination was considered important for mastitis detection because β-D-glucuronidase, the most significant enzyme in inflammatory processes, is released selectively.

The relationship between enzyme, presence of pathogens, and somatic cell counts was established in 220 milk samples obtained at random from individual quarters of apparently healthy udders of cows from four local dairy farms (Santiago del Estero and Tucumán, Argentina). Four of these samples were from cows of recent parturition and two from cows with severe clinical mastitis. Only 17% of the milk samples were normal with somatic cell counts 500,000 cells/ml or less. This ratio is the usual one throughout the area, and the remaining 83% showed higher somatic cell counts. Taking the latter as 100%, the presence of β-glucuronidase and the positive bacteriological analyses represented 76 and 74%, respectively.

INTRODUCTION

Inflammation of the mammary gland, commonly known as mastitis, is a complex disease in etiology, pathogenesis, sequelas, therapy, and other related aspects. Gross changes in milk composition induced by mastitis are well established; they are reflected in an increased number of somatic cells (3), progressive changes in ionic concentrations, and variations of the concentration of milk proteins such as casein, serum albumin (1, 2), immunoglobulins (4), and enzymes (6, 7). These modifications have been used to devise methods for mastitis detection. Some of these methods reflect the degree of udder infection fairly accurately, but somatic cell counting is the most acceptable and prevalent procedure. However, factors such as age, stage of lactation, parturition, stress (11), or vacuum fluctuations during machine milking (10) can influence somatic cell counts.

In clinical and subclinical mastitis, the afflux of mononuclear phagocytes [polymorphonuclear leukocytes (PMN) and macrophages] constitutes one of the essential body defenses against the disease. These cells close in upon lesions as a consequence of microbial stimulation, thus starting the phagocytic process with secretion of hydrolytic enzymes, which together with other factors participate in the degradation of tissue constituents in the inflammatory process. These enzymes can be nonlysosomal, such as lactate dehydrogenase and leucine 2-naphthylamilase, or lysosomes, such as β-galactosidase, N-acetyl-β-D-glucosaminidase, alpha mannosidase, and β-glucuronidase (14). All of them are secreted by stimuli that provoke an inflammatory response, but β-glucuronidase has proved to be the most significant selectively released enzyme in this process (12, 13).
Taking into account these facts, we have determined the relationship between the level of \( \beta \)-glucuronidase, measured qualitatively in whole milk, and the presence of mastitis pathogens and somatic cell counts. In this paper we also discuss the implications of the detection of \( \beta \)-glucuronidase as a means of mastitis diagnosis.

**MATERIALS AND METHODS**

**Collection of Milk Samples**

The samples, obtained from four local dairy farms, were taken at random from apparently healthy cows, without considering age or lactation stage, except for four cows of recent parturition. Only two samples were taken from cows with clinical mastitis.

Aseptic quarter milk samples were collected at the evening milking (12 h after the preceding one) with a previous water wash of the udders. All samples were cooled, transported to the laboratory, refrigerated, and stored until analysis.

**Bacteriological Analysis**

The standard method of milk dilutions was not used in the bacteriological analysis since our interest was focused on the isolation of pathogens and not on their quantification. We used one of the methods for the concentration of microorganisms [p. 58 in (9)] in cerebrospinal fluid (CSF).

Portions of milk samples were centrifuged for 10 min at 1,000 \( \times \) g (Rolco centrifuge model 2036) and then processed to isolate aerobic and microaerophilic bacteria. Only 10 \( \mu l \) was taken from the sediment with a calibrated loop and was spread over sheep blood agar and chocolate agar plates as well as in selective media for Gram-negative bacilli (McConkey's medium and eosin/methylene blue). The plates were then incubated at 37\(^\circ\)C for 24 to 48 h. At the same time, direct examination was carried out on Gram and methylene blue-stained preparations.

Identification of microorganisms was performed taking into account their cultural, morphological, and staining characteristics, as well as the results of the following biochemical tests: catalase, coagulase, sensitivity to novobiocine, fermentation of mannitol, hippurate, and bile aesculin hydrolysis, growth in BHI with 6.5% sodium chloride, oxidase test, and fermentative assays [p. 195 in (9)].

Identification of streptococci [p. 88 in (9)] was made by serological assay using the quick Slide Streptokit technique from Bio-Merieux Laboratory.

The isolated microorganisms were grouped into primary and secondary pathogens (16).

**Somatic Cell Count**

The somatic cell count was performed using the technique described for white blood globules (17). Milk samples were diluted 1/20 in Turk's solution (.05% methylene blue in 3% acetic acid) and counting was performed using a Neubauer chamber in a Zeiss microscope. The number of cells was determined according to Wintrobe (17).

**Identification of Somatic Cell Type**

One drop of whole milk was spread over a slide and air dried. It was stained with toluidine blue (.2% in water) from 3 to 5 s and immediately washed with water. Microscopic investigation was carried out with an immersion objective.

**\( \beta \)-Glucuronidase Assay**

\( \beta \)-Glucuronidase activity was assayed qualitatively using the synthetic substrate p-nitrophenyl-\( \beta \)-D-glucuronide (pnPG, Sigma) according to Stossel's technique (15).

The assay was performed with .8 ml whole milk, .2 ml pnPG (31.5 mg reagent and 100 \( \mu l \) Triton X-100 in 100 ml .05 \( M \) sodium acetate-acetic acid buffer, pH 5.). Incubation was effected for 18 h at 37\(^\circ\)C. The reaction was stopped by adding 1 ml .1 N sodium hydroxide. The degree of reaction was ascertained by measuring the intensity of the yellow color of p-nitrophenol liberated by the substrate. Positive degrees of the reaction were classified from + to +++.

**RESULTS**

Two hundred and twenty samples were studied; 17% corresponded to normal milks with somatic cell counts between 300,000 and 500,000 cells/ml, negative bacteriological cultures, and absence of the enzyme.
TABLE 1. Relationship between β-glucuronidase and the isolation of potentially pathogenic bacteria in milk samples with somatic cell counts higher than 500,000 cells/ml.

<table>
<thead>
<tr>
<th>Somatic count (cells/ml)</th>
<th>Samples</th>
<th>β-Glucuronidase-positive (%)</th>
<th>Potentially pathogenic bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>500,000–800,000</td>
<td>47</td>
<td>32 (+ to ++)</td>
<td>30</td>
</tr>
<tr>
<td>850,000–2,000,000</td>
<td>53</td>
<td>44 (+++)</td>
<td>44</td>
</tr>
<tr>
<td>Higher than 500,000</td>
<td>100</td>
<td>76</td>
<td>74</td>
</tr>
</tbody>
</table>

In the remaining 83% of samples, somatic cell counts were higher than 500,000 cells/ml. Considering this as 100%, the bacteriological analyses was positive in 74% of the samples and β-glucuronidase was detected in 76%, as shown in Table 1.

Identification of isolated microorganisms that are potentially pathogenic showed that most (43%) were *Streptococcus agalactiae*, a primary pathogen. The distribution of the remaining microorganisms is shown in Table 2.

When relating the high somatic cell counts in the samples with the presence of the enzyme, we obtained the results shown in Table 1.

Few neutrophils were observed in the differential cell counts in milk with somatic cell counts (300,000 to 500,000 cells/ml) and negative bacteriological cultures.

In samples with somatic cell counts higher than 500,000 cells/ml, 76% of the cases that presented positive enzyme reaction displayed a predominance of macrophages and PMN.

Six percent of the samples in which the enzymatic assay was negative and the culture positive showed a predominance of lymphocytes.

Table 3 shows all the results obtained in samples with counts higher than 500,000 cells/ml.

DISCUSSION

Among abnormal samples, those with somatic counts higher than 500,000 cells/ml (83%), a high correlation was observed between the presence of the enzyme and the isolation of bacteria: 76 and 74%, respectively. The reported difference of 2% corresponds to milks with positive detection of β-glucuronidase but negative bacterial cultures. This difference may be attributed to the effective action of phagocytic cells, followed by liberation of the enzyme, because the predominant cells are PMN. The production of the enzyme depends upon metabolically active cells and, once it is released, it is very stable, maintaining its activity for 2 mo at 30°C, thus allowing its detection in milks preserved at that temperature. Enzyme stability at 30°C does not cause false positives results in normal milks because β-glucuronidase enzyme is released only when the cells are stimulated.

No difference was observed between the presence of primary and secondary pathogens and the liberation of the enzyme, because the latter is a function of mononuclear phagocytic cells and not of germ pathogenicity, even though the highest percentage of isolation corresponds to *Streptococcus agalactiae*, a primary pathogen (Table 2).

When analyzing the correlation between high somatic cell counts and enzyme detection for a 100% abnormal milks, β-glucuronidase was present in only 76% of the samples. In these
TABLE 3. Relation between the presence of β-glucuronidase and bacteriological isolation in samples with somatic cell counts higher than 500,000 cells/ml.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Culture</th>
<th>Samples (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>74</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>6</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 total</td>
</tr>
</tbody>
</table>

samples, PMN and macrophages were identified microscopically, which was an expected result.

However, the number of somatic cells associated with enzyme activity was variable, because in milks with somatic cells slightly lower or higher than 500,000/ml, the presence of β-glucuronidase depended on cells previously stimulated by germs.

Six percent of the samples with high somatic cell counts showed positive bacteriological cultures and negative enzyme reaction. These results may have resulted from milk contamination, because the isolated bacteria were saprophitic, such as Staphylococcus epidermidis and S. saprobiticus. We believe, as already pointed out by other authors (5), that a positive culture of saprophitic germs does not always reflect abnormality; thus, it is necessary to determine whether this positive result corresponded to milk contamination or to infection of the udder or the mammary glands. Samples with severe clinical mastitis were included within this 6%, as expected, considering that the infection overcomes all natural defenses. In this case, the predominant cells were lymphocytes. However, we cannot discard the possibility that the 6% might be an error of false negatives.

In samples with high counts (between 500,000 and 800,000 cells/ml), 18% of them presented negative bacteriological cultures and negative enzyme reaction; this group included milk samples from four cows shortly after parturition. The remaining percentage may be attributed to lactation stage or stress, which may increase somatic cells (10). Samples were not selected but taken at random.

Although the determination of N-acetyl-β-glucosaminidase has been proposed as an indicator of udder infection (8), β-glucuronidase might be the most reliable indicator of an inflammatory process, since N-acetyl-β-glucosaminidase is liberated by other cells as in the case of epithelial tissue damage (6), which does not always correlate with the degree of infection.

The reaction of β-glucuronidase is very sensitive and can be detected in highly diluted milk samples (1/100, unpublished results).

We believe that this method of enzymatic reaction could be applied to mastitis detection in dairies. Because of its simplicity it can be carried out with high specificity in whole milks. The remarkable relationship between positive enzymatic reaction and bacteriological culture allows us to advocate the use of this method as a very effective diagnostic tool for subclinical mastitis.

REFERENCES