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# Detection of Antibiotics Residues in Raw milk Produced in Freha Area (Tizi-Ouzou), Algeria.

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**Abstract.** The misuses and uncontrolled veterinary drugs in animals food production can have harmful effects on consumers health and dairy industry. The presence of antibiotic residues in milk can cause partial or total inhibition of the growth of lactic acid bacteria, which can induce technological flaws on the cheese production. The aim of this study is to detect antibiotic residues in raw milk produced in Freha area (Tizi-Ouzou) in Algeria. A total of 171 milk samples were collected from 14 dairy farms and examined. The first screening of the samples was carried out by acidification test using *Bacillus stearothermophillus* (variety *calidolactis* ATCC 10149), followed by a confirmation test agar with spores of *B. stearothermophillus*, *B. subtilis* and *B. megaterium*. Our results showed a strong presence of antibiotic residues in raw milk, with 80 positive samples (46.78%). Most of them contained penicillin and/or tetracycline (88.75%), followed by macrolide and/or aminoglycoside (12.5%). In contrast, the sulfonamides were only present in 5% of the positives cases. The results indicate that most of the farmers do not always respect the time delay between the administration of antibiotics and the milk collection. Therefore, the control of antibiotic residues must be a major concern for producers and processors to protect health's consumers.

**Keywords:** antibiotics residues, raw milk, microbiology, acidification test, confirmation test**.**

## INTRODUCTION

Applied to milk, the term quality includes a range of concepts. It involves the proportions of main constituents, its chemical composition and organoleptic properties, the presence or absence of pathogenic germs, as well as exogenous substances, which can also affect the health's consumers (Ekman, 2000). Modern breeding farms use a wide range of veterinary drugs. These antibiotics are used either as growth promoters or as therapeutic remedies. Overuse and non respect of time between their administration in animal and the milk collection, may induce fortuitously milk contamination. Their presence in human foods is associated with several adverse public health effects, including hypersensitivity, gastrointestinal disturbance and neurological disorder. Another aspect of the problem is the fact that the presence of antibiotics in milk can prevent fermentation involved in the production of some basic foodstuffs such as yogurt and cheese (Rogister *et al.*, 2002).

Several methods have been described in the literature for the detection of antimicrobial substances in milk. Those differ in their sensitivity, their implementation and their cost. The control of antibiotic residues in milk can be checked after using a qualitative method such as microbiological screening methods and immunological tests but also with using a quantitative test as the analytical methods (liquid chromatography coupled with UV spectroscopy or mass

fluorimetry) (Pericas *et al*., 2010). The purpose of this study is to determine the incidence of antibiotic residues in raw milk produced in our area in order to have some information about the abusive using of veterinary drug in dairy cattle and to assess the risk for the health's consumers.

## MATERIALS AND METHODS

*Study area and sampling conditions:* This study involved a total of 171 individual raw milk samples collected from 14 dairy farms in Freha (Tizi-Ouzou area) in Algeria. All samples came from declared healthy cows by the farmer and not undergone any antibiotic treatment three weeks before. This milk is intended for human consumption or is delivered to different dairies. The raw milk samples were collected in sterile plastic bottles and sent to the laboratory in the same day, using refrigerated cooler and stacked ice-bag, stored at 4 °C. Before being tested, all raw milk samples were heat treated at 80 °C for 10 minutes in order to inactivate all inhibitory substances naturally present in the milk.

*Treatment of samples:* The detection of antibiotic residues in samples was performed according to the official European method for detecting antibiotic residues in milk (Commission Decision 91/180/EEC of 14 February 1991), which is applied in European Community since 1 January 2002 (the European 91/180.CEE, EC Regulation Nr. 1664/2006). Two tests were successively used: Acidification test based on the identification of a possible inhibition of *B. stearothermophillus* variety *calidolactis* ATCC 10149, as indicated by the turn indicator colored antibiotic residues that would be present in the sample, followed by a confirmation test, corresponding to the realization of three agar diffusion tests using tree bacteria strains: *B. stearothermophilus*, *B. subtilis* and *B. megaterium*.

*Acidification test:* This test allows an initial screening of all the samples tested. It is based on the addition in the milk sample of nutrient mixture with agar containing a pH indicator and spores of *B. stearothermophillus* (variety *calidolactis*, ATCC 10149, purchased from Pasteur Intitute from Algeria). Generally, the strain present a very good sensitivity and more particularly for penicillin. Normal growth and acid production by this organism after incubation causes a color change of the pH indicator (bromocresol purple) (SIGMA ALDRICH, Germany) which turns from purple to yellow. When milk contains inhibitory substances, the growth of the test organism is affected; the color of the pH indicator remains purple. A negative control (milk free from antibiotics) and a positive control (milk containing penicillin) are subjected to the test under the same conditions and at the same time as the tested samples.

*Agar diffusion test:* all positive milk samples or doubtful obtained by acidification test will be checked by confirmatory test. This test consists of using three agar diffusion tests inoculated with spores of *B. stearothermophillus*, *B. subtilis* and *B. megaterium*. The Muller-Hinton agar was previously melted at 100°C and cooled at 55 °C before being poured into Petri dishes. The medium was inoculated with $10^3$ to $10^5$ spores *B. stearothermophillus*, *B. subtilis* and *B. megaterium*/ml. After preparing a sterile paper disc, the latest were impregnated with milk samples and deposited on the agar surface. The Petri dishes were incubated at 55°C for *B. stearothermophillus* and 37°C for *B. subtilis* and *B. megaterium*. After 24h of incubation, the diameters of inhibition areas were measured by using calipers. The different families of antibiotics detected by inhibition of this microorganisms test are summarized in table 1.

## RESULTS AND DISCUSSIONS

The results of this study showed after using acidification test (first stage), the presence of antibiotics residues in 115 of the total 171 raw milk samples examined

(67.25%), reflecting their high presence in the milk collection. In contrast, the change of middle coloration from purple to yellow was only observed in 49 samples (28.65%) which were considered negative. It was impossible to confirm their presence in 7 milk samples (4.09%) (Table 2). The last samples were considered doubtful. That is the reason we needed a confirmation test.

Table 1

Detection of several antibiotic residues in raw milk samples

| Microorganism Test | Families antibiotics | Antibiotic |
|---|---|---|
| *Bacillus stearothermophillus* | Penicillins<br>Tetracyclines | Penicillin G<br>Tetracycline |
| *Bacillus subtilis* | Macrolides<br>Aminosides | Spiramycin<br>Erythromycin<br>Streptomicin |
| *Bacillus megaterium* | Sulfamides | Trimethoprim sulfamethoxazole |

Table 2

Results of analyzing raw milk samples after using acidification test

| Number of all samples examined | RESULTS | | |
|---|---|---|---|
| | Number of positives cases | Number of negatives cases | Number of doubtful cases |
| 171 | 115 (67.25%) | 49 (28.65%) | 7 (4.09%) |

In addition, the confirmation test showed that presence of antibiotics residues recorded only 80 against 91 raw milk samples examined, 46.78% vs 53.21% respectively (Table 3). The samples were considerate free from antibiotic residues.

Table 3

Results of analysis of raw milk after using confirmatory test

| Number of samples Examined | Results | |
|---|---|---|
| | Number and percentage of positive cases | Number and percentage of negative cases |
| 171 | 80 (46.78%) | 91 (53.21%) |

Table 4

Percentage per families of antibiotics residues in raw milk samples

| Number of positive raw milk samples examine | Result: (Number and percentage of positive cases) | | |
|---|---|---|---|
| 80 | Penicillins and/or Tetracyclines | Macrolides and/or Aminoglycosides | Sulfonamides |
| | 71 (88.75%) | 10 (12.5%) | 4 (5%) |

However, most positive raw milk samples showed a high percentage of contamination with penicillin and/or tetracycline (88.75%). Conversely, the macrolides and/or aminoglycosides were detected in only 10 over 80 positive samples (12.5%) and for Sulfonamides in only 5% of positive cases (4 over 80 positive samples) (Table 4). We noticed that only 10 over 71 samples contaminated by penicillin and/or tetracycline presented greater diameter of inhibition (more than 15±1mm) for *B. stearothermophillus*. The first screening test (acidification) revealed that 115 over 171 raw milk samples examined tested positive for

antibiotics residues. However this test was not able to conclude definitively the results of some samples qualified as doubtful, hence the need for a confirmation test. The agar diffusion technique, using the three strains, *B. stearo thermophillus*, *B.subtilis* and *B. megaterium*, has lifted any ambiguity for these doubtful samples. The choice of *B. stearothermophillus* as test strain is definitely better than the use of other strains such as *S. aureus*, which showed a lot of limiting factors such as in terms of susceptibility to antibiotics (low zone of inhibition at high concentrations of antibiotics), the risk of resistance (secretion of penicillinase by the microorganism or by mutation) and inhibition by fermenters germs (*Lactobacillus lactis*, *Streptococcus lactis*, *Streptococcus thermophillus*) (Hilan and Chemali., 1998). Indeed, *B. stearothermophillus* is characterized by a remarkable sensitivity to β lactam antibiotic; this growth is inhibited by 5 ppb of concentration of ampicillin. The sensitivity of the acidification test is particularly high for cloxacillin and reasonably acceptable for tetracycline.

The acidification test is thus characterized by sensitivity close to MRL for cloxacillin and 50-100% of MRLs for tetracycline compared to the old method using *Streptococcus thermophillus* as test microorganism, that offers to both antibiotics mentioned above that sensitivities of 3 to 4 times the MRL and 2-4 times MRL (Navratilova, 2008).

The risk to have false negative results with milk at concentrations of antibiotic residues near very low for MRLs is thus limited, thus the confirmatory test shows a high specificity. Our results were not in agreement with those reported by Ben Mahdi and Ouslimani (2009). In fact, their results indicate a low level of contamination rate (9.87%). This difference could be explained by the relatively small number of our samples. The results were similar to those reported by Zinedine *et al.* (2007) who obtained 57.14% positive cases. However, the percentage of presence of antibiotic residues in raw milk varied in some studies. In fact, Srairi *et al (*2004), Kivaria *et al* (2006), Adesiyun *et al* (2007) and Kouame *et al* (2010) have obtained 25, 7, 6.5 and respectively 24.7% positive cases.

The high percentage of contamination of the milk by inhibitors such as antibiotic residues, can be probably explained mainly by massive and uncontrolled intra-mammary pharmaceutical preparations for the treatment and prevention of bovine mastitis, not respecting the waiting times after treatment and secondly by a voluntary addition of germs growth inhibitors (antibiotics, antiseptics) in order to stop microbial growth and stabilize the microbial quality of milk (Zinedine et *al*., 2007). The high contamination of milk samples in tetracycline and/or penicillin was also confirmed by Ben Mahdi and Ouslimani (2009) who reported their presence in 97.33% raw milk samples. According to Ameur *et al*. (2008), in our area and other part of Tizi-Ouzou area like Azazga and Yakouren, the use of intra- mammary syringes is often aiming to prevent acute mastitis. The most prescribed and used products contain tetracycline, penicillin and rarely mcrolides. The choice of these molecules is mainly based on their efficiency and their low price.

In addition to the risk to public health (allergic reactions, influence on the intestinal flora and the risk of emergence and increase of antibiotic-resistant strains), the antibiotic residues represent a real problem for milk processors because of their negative impact on the process of lactic fermentation. In fact, lactic acid bacteria like *Streptococcus thermophillus*, *Lactobacillus bulgaricus* and *Lactococcus lactis* can play an essential role as starter by acidifying the milk, there by resulting in the precipitation of milk protein (casein), the development of aromas linked to proteolytic activity and lipolytic lactic strains and the inhibition of the growth of alteration microorganisms or potentially pathogenic bacteria such as coliforms, *Pseudomonas*, *S. aureus* and *Listeria monocytogenes* (Chamba, 2008). The presence of antibiotic residues in the raw milk may partially or completely inhibit the growth of lactic acid bacteria involved in the development of dairy products such as cheese and yogurt, leading to manufacture accidents. The most common accidents are the milk

coagulation defects, the inadequate draining and the risk of uncontrolled proliferation of gasifier's germs, insensitive to antibiotics, such as coliforms, *Bacillus*, *Clostridium* and *Proteus* (Berger and Lenoir, 1997). This problem generates economic losses every year.

## CONCLUSION

The results of this study indicate clearly the high contamination of our raw milk samples by antibiotic residues. This can be explained by the intense and misuse of veterinary drugs, as well as the non respect of time between the administration of the antibiotic to patient and the milk collection. To solve this situation, certain measures must be applied, such as: the respect of good veterinary practices, the respect of withdrawal time before the collection of milk product, and a regular and systematic screning of antibiotic residues in milk before consumption or technological use. Finally, we hope to lead other studies in the identification of antibiotic residues in milk, using a combination of two techniques: the microbiological method with agar diffusion and the analytical method (high performance liquid chromatography) to determine not only the type of antibiotic residues used but also their concentration in the milk collected.

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