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Comparative Analysis of Antibiotic residues in Local and Imported Milk by Microbiological Inhibition Test in Constantine Region (North East Algeria)

Boultif Latifa*[1], Chebira Bassem[1], Abdeldjelil Mohamed Cherif[1], Djenna Djahida[2] and Redjal Ismahane[3]

[1]*PADESCA Laboratory, Institute of Veterinary Science, University of Mentouri – Constantine Algéria*
[2]*Direction of Agricultural Service – Constantine, Algeria*
[3]*Numidia Company, Giplait group, Constantine, Algeria*

ABSTRACT

In this study, the authors have conducted a search of antibiotic residues in commercialized milk in Constantine region (North East Algeria). In order to assess the level of contamination in the region, because no control is applied until now. Sampling concerned cow's milk produced locally as well as imported milk powder. The search was conducted using Delvotest®, a microbiological inhibition test for a qualitative detection of antibiotics residues, especially β -lactams antibiotics. A total of 180 samples were analysed: 120 samples of local milk and 60 samples of imported one. Statistical analysis will focus on the percentages of contamination which will be compared by applying chi-square tests (χ2). Results showed that 40% of milk samples produced locally are contaminated with antibiotics residues. While contamination in imported milk is much lower (5%). Statistical analysis showed a significant difference in contamination levels between local and imported milk samples. These results should urge local authorities to establish an effective control of the entire dairy industry to prevent potential risks caused by antibiotics residues.

Key words: antibiotics, residues, milks, Constantine, Algeria.

INTRODUCTION

In Algeria and other North African countries, antibiotics are among the most used molecules in cattle. Furthermore, in Algeria, milk is a complete food witch widely consumed and occupies a prominent place in the diet. It is even the main source of animal protein [1].

Because of the insufficient local milk production, the country is forced to import large quantities of milk powder to meet population needs. Making Algeria, the second milk powder importer in the world [2].

In order to protect consumers against potential effects caused by residues, a rigorous monitoring is required on both local and imported milk. Indeed, residues present in milk may be involved in several health problems (antibiotic-resistant [3-6], allergic problems [7-10], immune imbalance and development of some cases of cancer [11,12]) as well as causing economic losses to the dairy industry [12-17].

The aim of this study is to detect antibiotic residues in commercialized milk in Constantine region (North East Algeria); because no control is applied until now.

Sampling concerns locally produced milk as well as imported milk in powder form. The residues presence is qualitatively evaluated through the microbiological inhibition test "Delvotest®".

The search will concern mainly β -lactams antibiotics, because they are the most used ones by intra-mammary route for the treatment of udder diseases [18]. This route of administration seems to represent the main cause of milk contamination by antibiotic residues [19-21].

MATERIALS AND METHODS

2.1. Equipment Used:

2.1.1. The Delvotest® Kit

Delvotest® T is a standard diffusion test for the detection of residues of antibacterial substances (antibiotics and sulphonamides) in milk. The Delvotest® kit contains 100 ampoules containing a solid agar medium seeded with a standardised number of spores of *Bacillus stearothermophilus* var. *calidolactis* together with required nutrients for growth purposes. The medium is coloured purple by the pH indicator bromocresol purple. The kit contains also a preset 0.1 ml dosing syringe and disposable tips for sampling.

2.1.2. Delvotest® Incubator

It is a specially designed apparatus for incubating Delvotest® ampoules, with an incubating temperature set at 64 ° C.

2.1.3 Sampling

2.1.3.1 Cow Milk

Milk samples were collected over a period of one year (from March 2013 to June 2014).

A total of 120 samples were collected throughout the 12 municipality of Constantine region (North-East Algeria), with 10 samples for each municipality.

The collected milk is a mixture of milk taken from bulk milk tanks. Each milk sample is kept in a properly labeled and sealed sterile plastic bottle. The samples are then sent to the laboratory in insulated tanks where they are stored in a freezer until their analysis. The freezing process does not alter the antibiotic concentrations as reported by many authors [22,23].

2.1.3.2 Milk Powder

Milk powder sampling concerned three of the most consumed milk brands in the region. A total of 60 samples have been used for antibiotic residues search.

Sampling is done in sterile sealed plastic bags, stored in the refrigerator until analysis.

To be analyzed, the milk is reconstituted according to manufacturer's recommendations.

METHODS

2.2.1 Analysis Protocol

After thawing the samples in a water bath at the temperature of 15 ° C for one hour (Romnée, 2009), 0.1 ml of each milk sample is slowly deposited on the agar of the corresponding labeled ampoule. Results are read after 2:30 to 3:00 hours of incubation at 64 ± 0,5 ° C. Reading is interpreted on the color transfer basis. Originally the medium in each ampoule is coloured purple. Milk samples which are free from antibacterial substances, or contain them below specified levels will, when added to the ampoule test and incubated, allow germination and growth of the bacteria. This will lead to a change in colour of the indicator from purple to yellow. When the milk sample contains antibacterial substances at or above the test sensitivity, growth is inhibited and the colour remains predominantly purple.

2.2.2 Statistical Analysis

Comparison between contamination levels of locally produced milk and imported milk powder was made using chi-square tests ($\chi 2$). After displaying data in a contingency table, the $\chi 2$ statistic was calculated using the formula:

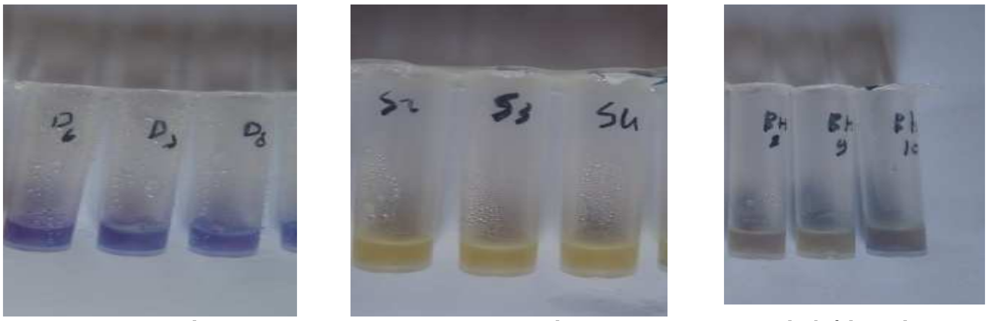
$$\chi^2 = \sum \frac{(Oi - Ei)^2}{Ei}$$

where :
Oi : the observed counts in the i^{th} cell of the table .
Ei : the expected counts in the i^{th} cell of the table.
Any value of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

3.1. Expression of Results

After incubation, results are read based on color change as shown in figure 1, where purple indicates a positive result, yellow color indicates a negative result, while a color between the two is said " doubtful result".



Positive results   Negative results   doubtful Results

Fig. (1) Examples of positive, negative and doubtful results after analysis by the Delvotest®

3.1.1 Cow Milk

25% (30 from 120) of the analyzed samples were positive, 60% (72/120) were negative and 18 out of 120 (15%) were doubtful (Figure 2).

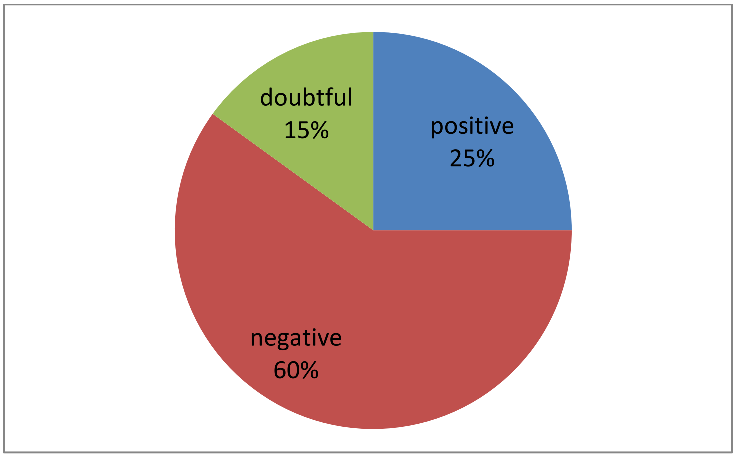


Fig. (2) cow's milk contamination percentages revealed by the Delvotest®

Frequency of each result (positive, negative or doubtful) for each studied municipality is shown in Table 1. Results show heterogeneity of contamination cases between the different municipalities, some of them have no contamination at all, such as M11 and M12. While others, present higher contamination level, with a frequency of positive results reaching 0.7 for M6 and M4 and 0.6 for M3 (table 1).

Table. (1). Frequency of positive, negative, or doubtful results by municipality

municipality	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12
Frequency of positive samples	0.2	0.1	0.6	0.7	0.4	0.7	0	0.2	0.1	0	0	0
Frequency of negative samples	0.7	0.8	0.4	0.3	0.3	0.2	0.6	0.6	0.7	0.7	1	0.9
Frequency of doubtful samples	0.1	0.1	0	0	0.3	0.1	0.4	0.2	0.2	0.3	0	0.1

3.1.2. Milk Powder

Concerning powdered milk, from a total of 60 samples, only 2 were positive (3.3%), 57 were negative (95%), and one was doubtful (1.6%) (Figure 3).

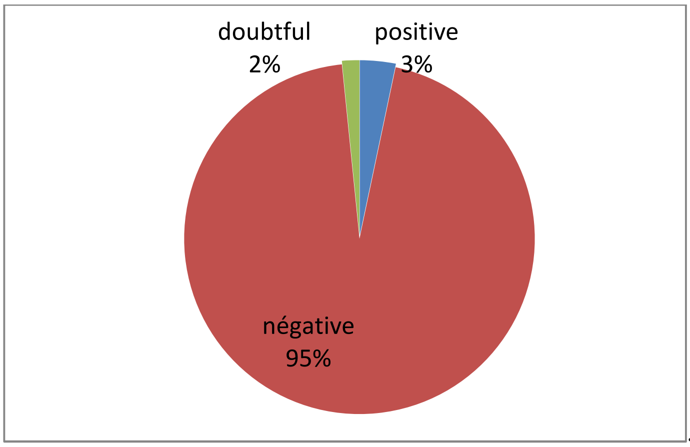


Fig.(3) Milk powder contamination percentages revealed by the Delvotest®

3.1.3. Statistical Comparison of Results Between Local and Imported Milk

Results of chi-square test ($\chi2$) show a significant difference in contamination levels between local and imported milk samples. Confirming the high contamination levels in locally produced milk (Table 2).

Table .(2). Statistical comparison between contamination levels of local and imported milk

	positives		Negative		Doubtful		total
	(observed)	(expected)	(observed)	(expected)	(observed)	(expected)	
Cow milk	30	21,33	72	86	18	12,6	120
Milk powder	2	10,6	57	43	1	6,33	60
Total	32		129		19		180
The test statistic is: $\chi 2$ = 23.8 with 2 degree of freedom							

The degrees of freedom is (r-1) (c -1) = 2 r= # rows c= # of columns

From the chi-squared table:
At 2 degree of freedom and an alpha level of 0.05 χ^2= 3.84
At 2 degree of freedom and an alpha level of 0.01 χ^2= 9.21

Our test statistic $\chi 2$ is equal to 23.8, it exceeds largely the values the table gives both at 5% and 1% risks, which means the existence of a significant difference between contamination levels of the two milk samples,

DISCUSSION

Many rapid and selective microbiological inhibition tests are applied for the detection of residues. Tests are used at the farm level, dairy plants and approved laboratories [24-27]. The Delvotest® is one of the first microbiological inhibition tests used directly on bulk tank milk, for the detection of antibiotic residues. This method was advocated since 1982 by the Association of Official Analytical Chemists[28,29].This test uses a bacteria strain particularly sensitive to many antibiotics, including penicillin. It is a specific test for the detection of β -lactam antibiotics (2-4 mg / kg), but appears to have a decreased specificity to other molecules such as tetracycline (200-400 mg / kg) macrolides (neomycin, erythromycin), streptomycin, gentamicin and chloramphenicols [30,31].

The bacterium used by the test is *bacillus stearothermophilus* variety *calidolactis* which is widely used in dairy industry, particularly in the production of yoghurt.

Owing to its sensitivity to many antibiotics, the use of this thermophilic bacterium, successfully permits the detection of antibiotic residues in milk and its derivatives [32], Which explains its use in the Delvotest® as a qualitative method for the detection of antibiotics residues.

It must be pointed out however, that this method has some drawbacks: it does not allow specific identification of antibiotics, which makes it merely a qualitative method. Its incubation period is quite long (2.5 to 3 hours) and its sensitivity is marked for β-lactam antibiotics essentially penicillin, but apparently less for other antibiotics.

In local milk samples, contamination is quite important: 40% of the analyzed samples contain residues of antibiotics including 25% positive and 15% doubtful ones.

Comparison of our results with other studies: shows that the positive percentage of (25% found in our study concurs with some works done in Algeria. Like the one of Aggad et al. [33] where 28% of the milk samples were positive.

These results are also similar to those of other authors such as Guetarni [34] with 26.38 % and Srairi et al [35] with 26% found in a Moroccan study.

Some other studies report higher percentages of contamination than ours. These are mainly observed in countries where milk control is not regular. In such countries, the percentages of positive samples are much higher and vary considerably [36].

Like in China, where during 2002 and 2003 the residue control revealed 37% positive results in bulk milk and 17.24% on sterilized milk (UHT) [37]. In Pakistan, 36.5% of milk marketed in 2006 was contaminated by β-lactams [38].

Furthermore, investigations in Brazil show that almost 50% of commercialized pasteurized milk was contaminated with antibiotic residues [39].

Such high percentages were also observed in eastern European countries, where in Poland, Rybinska et al. reported 13-22% of positive results [40]. And in Montenegro, where 7.84% of samples are positive [41].

In other parts of the world, the reported contamination percentages are medium, such as in some African countries where, 14.9 % of samples are contaminated in Kenya [42] and 8.5 % in Ethiopia [43]. In Colombia, Díez et al. showed that 12.8 % of samples are positive [44]. In some parts of Brezil, Borges et al. reported 4.3 % of positive samples [45]. In India, Sudershan and Bhat found 9 % of positive samples [46].

In Turkey, Ceyhan and Bozkurt found 5.5 % of positive samples in their study conducted in Ankara on 200 samples [47].

In European Union countries, the percentages of positive results are very low reaching less than 0.5 % in Western European countries [48].

For example, in Belgium and Denmark, they are 0.1% [49], in Spain they are 0.18% [50] and in Sweden, percentages are between 0.08-0.26 % [51]. Such results reflect long years of efforts to control residues in milk. In England and Wales for instance, residue control is practiced since 1965.

In some Eastern European countries, the percentages are relatively low. In Croatia, only 0.4 % of samples were positive [52]. In the Czech Republic the percentage of contamination is 0.5% and in Lithuania, 0.8 % [53,54].

By analyzing the values mentioned above, it appears that contamination percentages are much higher in developing countries in Asia and Africa (including Algeria) whereas in Europe, the percentages are significantly low.

In fact, countries where strict milk control is practiced regularly for a long time, contamination levels are minimal; however, in countries where control is occasionally done, contamination is very important.

Given the risks on consumers' health and the losses suffered by dairy processing industries, data advanced by our study, should be considered as a warning sign, to implement strict controls throughout the Algerian dairy industry.

Concerning powdered milk, studies are limited compared to those of cow's milk. Our results (3.3 % positive samples) are lower than those reported in Mexico by Tolentino et al. In their study on four milk brands (A, B, C, D), Results showed respective milk contamination percentages of 47.2 %, 58.3 %, 44.7 % and 50% [55].

CONCLUSION

Analysis of antibiotic residues by Delvotest®, of milk samples produced locally, shows a high level of contamination compared to reported levels in some other countries.

These contamination levels are however, significantly much lower in imported milk.

The entire local milk production industry should be carefully monitored to prevent any possible risks caused by antibiotics residues on both consumer and dairy industry.

Strengthening border control of imported milk products should also be considered.

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