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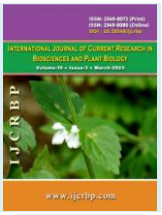

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Determination of antibiotic residues in raw cow's milk sold in two dairies in the Koulikoro Region using the HPLC technique

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Abstract

Veterinary treatments, mainly antibiotics, used for therapeutic or prophylactic purposes in dairy farming can be the cause of the presence of antibiotic residues in milk. However, these residues constitute a major health concern for consumers. For the dairy industry, whose objective is to have a raw material suitable for processing, it is necessary to screen for antibiotic residues in milk at each collection. This study is based on two parts, a survey of seventy people (10 practicing veterinarians and 60 milk producers) to describe the main molecules of antibiotics used in dairy cattle farming in the two basins of Tienfala and Kasséla in the Koulikoro region, and a search for residues by Delvotest SP with confirmation of positive samples by liquid chromatography (HPLC). To this end, sixty (60) samples of raw milk were collected in two sampling campaigns per site carried out 15 days apart. All samples were analyzed using the Delvotest SP rapid detection of antibiotic residues in milk kit first before being analyzed by liquid chromatography (HPLC) for positive samples. The results of the surveys revealed the predominance of three pathologies within dairy herds: diarrhoea, mastitis and pulmonary infections with a combination of several antibiotics belonging to five (5) different families of antibiotics. Of the 60 samples submitted for analysis, 38 were found to be positive with Delvotest SP, i.e. a contamination rate of around 63.33%. Of the 38 samples positive to the rapid test, 26 responded positively to confirmation by liquid chromatography, i.e. a rate of 68.42% with mainly four antibiotics belonging to the families of β-lactams and tetracyclines. The results of the study effectively prove the effective presence of antibiotic residues in raw cow's milk from the mini-dairies of Kassela and Tienfala. They thus characterize current practices in the treatment of dairy cattle with antibiotics in the farming areas studied. Compliance with withdrawal periods with the elimination of milk from treated cows must be obtained by raising the awareness of producers by animal health professionals.

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Introduction

The Malian livestock has been estimated since 31/10/2017 at 11,415,900 cattle, 17,400,000 sheep, 24,023,800 goats, 1,192,900 camels, 561,500 horses, 1,099,900 donkeys, 84,150 pigs and 45,023,800 poultry in 2017 (DNPIA, 2017). Estimates are made on the basis of data from the 1991 national livestock census, to which average annual growth rates of 3% for cattle, 5% for sheep/goats, 2% for horses, 2% for donkeys, 2% for camels and 1.2% for pigs. Poultry numbers are obtained through estimates made by DNPIA agents at the regional, local and municipal levels (DNPIA, 2017). Livestock represents around 10% of the national GDP and contributes 18% to the income of agro-pastoralists and 80% to that of populations in exclusively pastoral areas. The country's third export revenue after gold and cotton, livestock farming is far from covering national milk needs. Indeed, Mali spends every year between 10 to 15 billion FCFA for the importation of milk and dairy products, despite its significant animal resources. In Mali, the milk available, i.e. the quantity of milk used (consumption, sale, donation, etc.) by the breeder remains low to very low (890,484 tonnes), i.e. 44 to 50% of the milk potential national (DNPIA, 2017).

As for the share of the average level of milk consumption per capita on the basis of what is available, it is around 44 liters per capita and per year (INSTAT, 2015) a figure still far from the 62 liters as the standard of the CAM for an adult person. Antibiotics in Mali remain among the most widely used molecules in cattle breeding. Their use, as a curative or preventive treatment or as a supplement in animal feed, inevitably leads to the presence of residues in the foodstuffs derived from these animals. Today, the problem caused by antibiotic residues is to be feared because the quantities of fresh milk reserved for processing are still insufficient to afford to reject milk containing antibiotics (Boultif, 2014). The most commonly used antibiotics are tetracyclines, penicillins and cephalosporins administered parenterally (Reybroeck, 2010). The presence of antibiotics in milk is a limiting factor for mini yoghurt dairies because they inhibit the fermentation process (Heeschen and Bluthgen, 1990). Faced with these risks, several countries have regulated the use of antibiotics and initiated the systematic control of raw milk before its use. The general objective of the study was to determine the content of antibiotic residues in cow's milk produced in sites of two dairy basins (Kasséla and Tienfala) in the Koulikoro region.

Materials and Methods

Study area and period

The prospecting study took place in 2020 and in October and November. Sampling took place at two sites (Kasséla and Tienfala), each with a mini-dairy and located in the Koulikoro region (Fig. 1). They serve as supply sites for milk and dairy products for the inhabitants of the city of Bamako.

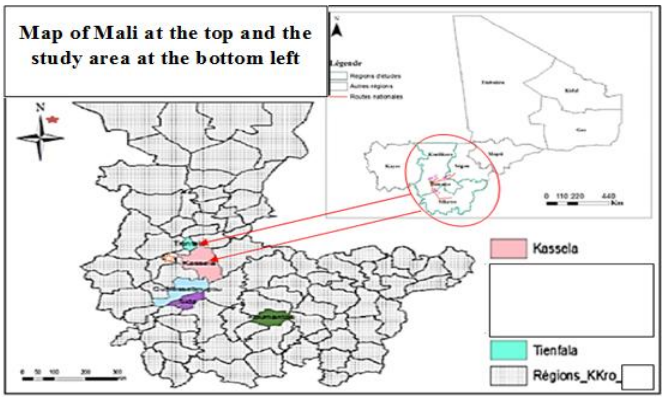

Fig. 1: Study area and sites.

The biological material of this study consists of samples of fresh raw cow's milk. Milk samples were taken at sites organized into mini-dairies, representing the two main dairy basins in the Koulikoro region. For each of the two sites, four sampling points (farm, collection center, retailers and mini-dairy) were selected and six composite samples of raw milk (farms (1), collection centers (1), retailers (1) and (2) for the mini-dairy) of a total volume of 50 mL each were collected (2 sites x 30 samples per site). The samples (n=60) all taken the mornings maximum 1 hour after the milking of the cows, in glass bottles, were labelled and placed under refrigerating plates. They were then transported to the laboratory 2 hours later after the samples were taken, where they were kept at 4°C before the analyses.

Methods of analysis for antibiotic residues

The samples were processed at the Central Veterinary Laboratory (LCV) and tested using two (2) methods (a rapid test and a confirmation method). All samples were first subjected to the rapid test (Delvotest SP) then all positive samples were analyzed with the confirmation method by high performance liquid chromatography (HPLC). The latter involves four (4) steps: extraction of antibiotic residues, concentration, purification and data analysis.

Rapid detection of antibiotic residues by the Delvotest SP

For the detection of antibiotic residues in milk, the known and validated commercial test Delvotest SP was used (Fig. 2). The Delvotest SP is a broad-spectrum microbiological selection test, allowing the detection of residues of anti-infective substances in milk at levels close to the maximum residue limits. It is particularly sensitive to penicillins, cephalosporins and sulfonamides (Reybroeck, 2004; Verhnes and Vandaele, 2002). The test comes in the form of ampoules containing an agar medium inoculated with the test germ (spores of *Bacillus stearothermophilus* var. calidolactis), with a colored pH indicator, trimethoprim and tablets of nutrient medium to be incorporated into the ampoules at the time of their use. It is a simple, standardized biological test, based on the search for inhibitors in raw milk by the multiplication of a germ: *Bacillus stearothermophilus* var. calidolactis (Zinedine et al., 2007). It is not specific but offers a wide detection spectrum and good sensitivity to penicillins. Its main drawback is its incubation period of 2h30 to 3h (Brouillet, 2002), (Verhnes et al., 2002). Contact with the milk sample takes place directly in the liquid culture medium. The experimental protocols then provide for prior heating of the sample to be tested in order to inactivate the natural inhibitors of milk. A sample of 0.1 ml of milk is left to diffuse in the agar medium, the ampoule is closed with adhesive tape and placed for 2h30 to 3h in a small incubator at 64°C plus at least 1°C.

In the presence of inhibiting substances, the color of the agar medium remains purple (violet) because they prevent the growth of the germ and therefore the production of acid (+Positive). If the milk does not contain any inhibiting substances, the pH indicator changes from purple to yellow due to the production of acid by the germ (-Negative). The results are read using the images shown in Fig. 3.

Fig. 2: Delvotest Kits and Incubator.

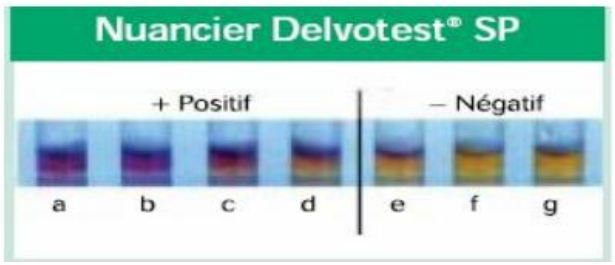

Fig. 3: Expression of Delvotest results.

Confirmation of the presence of antibiotic residues by HPLC

The Delvotest SP positive samples were subject to identification and quantification of certain residues by a physico-chemical method. Confirmation was carried out using a high performance liquid phase chromatograph (HPLC) equipped with a column (Lithosphere 100-RP-18 5µm in reversed phase: 4.6 x 250mm) and equipped with a ultraviolet (UV) visible detector to quantify the concentration of antibiotic residues. Before being identified and quantified, the antibiotic residues contained in the milk samples were extracted according to the protocol below (is it the protocol?). The residues sought were chosen to correspond to the most commonly used antibiotics in the field, in particular β-lactam antibiotics (amoxilline, ampicillin, penicillin G) and a sulfonamide (sulfisoxazole). This method involves the extraction of samples with organic solvents, rinsing by solid phase extraction (SPE) and derivatization before analysis by HPLC. The method involves the determination of residues of three β-lactams [Amoxilline, Ampicillin and Penicillin G] and Oxytetracycline in cow's milk at levels ranging from 0.5 to 100 ng/g.

Preparation of standards and solutions

a) Stock standard solutions (2000 µg/ml)

Place 20 mg of each analytical standard in a 10 ml volumetric flask and fill to volume with MeOH (for Amoxillin, Ampicillin and Penicillin G) and MeCN (for Oxytetracycline).

Store all standard solutions at -20°C.

b) Mixture of standard solution (50 µg/ml)

Put 250 µl of each standard solution (2000 µg/ml) in a 10 ml volumetric flask and fill to volume with MeCN.

c) Standard solution mixture (250 ng/ml)

Put 50 µl of standard solution mixture (50 µg/ml) in a 10 ml volumetric flask and fill to volume with MeCN.

d) Working standard solution

Put 5 µl, 10 µl, 50 µl, 100 µl, 300 µl and 600 µl of standard solution mixture (250 ng/ml) in six 10 ml volumetric flasks and fill to volume with MeCN to obtain solution mixtures working standard of 0.5 ng/ml, 1 ng/ml, 5 ng/ml, 10 ng/ml, 30 ng/ml and 60 ng/ml, respectively. Table 1 summarizes the process for preparing standard solutions.

e) Enriched samples

Add 10 µl, 20 µl and 500 µl of standard solution mixture to a 2.5 g sample in a test tube, let stand while continuing the sample extraction procedure.

Table 1. Preparation of analytical standard solutions.

Level	Volume (µl) of standard solution mixture (250ng/ml)	Final volume (ml)	Concentration of standard solution (ng/µl)	Dilution solution
1	5	2.5	0.5	MeCN
2	10	2.5	1	MeCN
3	50	2.5	5	MeCN
4	100	2.5	10	MeCN
5	300	2.5	30	MeCN
6	600	2.5	60	MeCN

Table 2. Chromatography conditions.

Chromatography column	Reverse phase C18, 4.6 x 150 mm, 3.5 µm
Mobile phase	MeOH:H_2O (97:3, v/v),
Mobile phase flow type	Isocratic
Debit	1 ml/min
Injection volume	50µl
Sample temperature	25°C
Column temperature	30°C
Reading time	9mn
Detector	DFL2475
Excitation wavelength	365nm
Emission wavelength	465nm

Preparation of milk samples

a) Place a 2.5 g sample of milk in a 50 ml centrifuge tube.

b) Add 10 ml of MeCN for the first extraction, vortex for 30 seconds and centrifuge at 2500 rpm for 3 min. Transfer the supernatant to another 50 ml centrifuge tube.

c) Extract again with 5 ml of MeCN. Combine the extract and mix with 20 ml of H2O and 40 µl of triethylamine.

d) Rinse the extract with an SPE C18 cartridge conditioned with 5 ml of MeCN then 5 ml of MeCN:H_2O (40:60, v/v) containing 0.1% of triethylamine; vacuum for 5 min.

e) Wash the cartridge with 3 ml of hexane and place under vacuum for 5 min. Elute the residue with 10ml of MeCN in a 12.5 ml amber vial. Evaporate the eluate to dryness under nitrogen at 600°C.

Derivatization

a) Dissolve the residue in 1 ml of MeCN, put it in an ultra-sonic bath for 20 min then add 100 µl of N-methylimidazole and 100 µl of trifluoroacetic anhydride.

b) Allow the sample to stand protected from light for 35 min before transferring an aliquot to an autosampler vial and injecting into the HPLC-DFL.

Chromatography conditions

a) System validity and critical conditions to assess system validity, inject at least five replicates of an intermediate standard used for the calibration curve. The relative standard deviation (RSD) of the maximum response and the retention time (TR) must not exceed 5%.

b) Run the HPLC under the conditions summarized in Table 2.

Results

Survey results

Results with practicing veterinarians

The analysis of the results of the survey carried out among ten (10) practicing veterinarians in the area of the mini-dairies of Kasséla and Tienfala made it possible to highlight several pathologies in dairy cows in the region. Digestive and metabolic disorders (mainly diarrhoea) remain the dominant pathologies, followed by breast diseases (mastitis) and pulmonary infections.

This survey of practitioners shows the use of a combination of antibiotics belonging to at least five (5) families for the treatment of the various pathologies described**.**

Results with producers, collection centers and mini-dairies

The analysis of the results of the survey carried out among sixty (60) producers showed the strong demand from veterinary practitioners for diagnosis and treatment (83%). However, a rate of 42% is totally unaware of waiting times, those who know about them do not use any sign to identify treated animals, even fewer written documents. The producer simply uses his abilities to visually recognize his animals and memorize the approximate dates of different events. For milk recording, none of the producers use screening tests for antibiotic residues and consume or sell their milk without dosage.

Screening by the Delvotest SP

The results of the search for inhibitor residues by the Delvotest SP method are presented in Table 3.

Table 3. Detection of inhibitors in raw cow's milk by the Delvotest SP.

	Number of samples analyzed	Results			
		Positive		Negative	
		Number	%	Number	%
Total	60	38	63.33	22	36.67

The results show that:

Thirty-eight (38) milk samples out of 60 tested responded positively to the Delvotest SP, i.e. a rate of 63.33% of positive samples.

Twenty-two (22) milk samples out of the 60 tested did not react, i.e. a rate of 36.67% of negative samples.

These results are represented by the graph below:

The search for antibiotic residues in milk by the Delvotest SP showed that thirty-eight (38) positive samples, i.e. 63.33%, are contaminated with antibiotic residues (through the reaction of inhibitory substances) and that twenty - two (22) negative samples, i.e. approximately 36.67%, did not contain any of these substances. The thirty-eight (38) positive samples were then subjected to the confirmation test by liquid phase chromatography, the results of which are summarized in Table 4.

Confirmation par méthode quantitative HPLC

The results of the HPLC confirmation tests of the presence of antibiotic residues detected in 38 of the Delvotest SP positive samples (microbiological method) are presented in Table 4.

The confirmation results show that:

Twenty-six (26) milk samples out of 38 tested responded positively to HPLC analysis, i.e. a rate of 68.42%.

Twelve out of 38 milk samples are not contaminated, accounting to 31.58% (Table 5) Concentrations of antibiotic residues sought in raw cow's milk.

Table 4. Overall results of confirmatory HPLC tests.

	Number of samples	Results			
		Positive		Negative	
		Number	%	Number	%
Total	38	26	68.42	12	31.58

Table 5. Antibiiotics in cow's milk.

Families antibiotics	Residues research	Concentration (µg/kg)		LMR (µg/kg)	Superior to LMR	
		Minimum	Maximum		Number	Taux (%)
β-lactams	Amoxycillin	61.79	381.95	4	9	34.62
	Ampicillin	12.16	69.13	4	7	26.92
	Pénicillin-G	97.64	368.85	4	17	65.38
Tetracyclines	Oxytetracyclin	127.26	334.53	100	21	80.77

The concentrations of most of the molecules tested are above the maximum residue limits (MRL). The dominant β-lactam residue is Penicillin-G (65.38%), Ampicillin presented the lowest residue and Oxytetracycline was the most dominant antibiotic of all molecules (Fig. 4).

Discussion

The concentrations of antibiotic residues recorded by the present study remain similar to the proportions of 70% reported in Kosovo by Sulejmani et al., (2012), 89% and 97.3% found in Algeria respectively by Tarzaali et al., (2008) and Ben-Mahdi et al., (2009); indicating that in the case of Mali, the antibiotics could likely come from both current and past misuse.

The results of the present study on antibiotic residues revealed that antibiotics contaminate raw cow's milk indicating a possible wide use of the latter in Mali.

However, the levels of ATB residues in the milk samples tested proved to be non-negligible in most milk samples, as the concentrations were above the MRLs set by the Commissions of the European Union (EU, 2015; WHO, 2015).

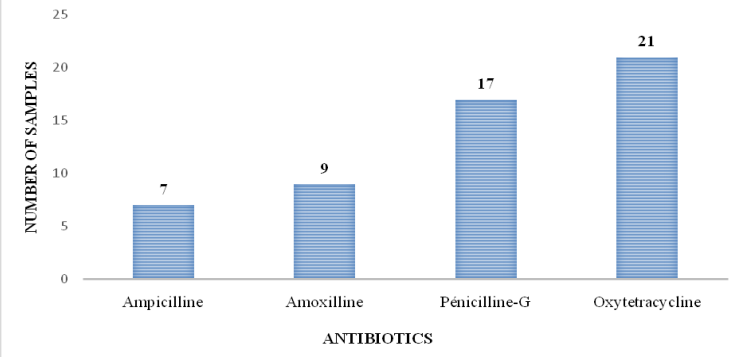

Fig. 4: Illustration of the frequency of detection of antibiotics in milk.

Inappropriate use of ATB products is therefore one of the potential risk factors for exposure of milk producers and consumers to ATBs. The same observation was made in Benin (Ahouangninou et al., 2011), Senegal (Cissé et al., 2003), Ghana (Ntow et al., 2006; Bempah et al., 2011) and Côte d'Ivoire (Doumbia and Kwadjo, 2009). The big problem with ATBs in West Africa lies in their free marketing (Ahouangninou et al., 2011).

Conclusions

The systematic search for antibiotic residues in milk samples remains the only means of prevention that can ensure the complete safety of this product. To do this, it is therefore imperative to have reliable, sensitive and specific detection methods. The results of our survey carried out in the field with practicing veterinarians

show, on the one hand, that the beta-lactam and tetracycline families remain the most used in the treatment of dairy cattle facing various pathologies. And on the other hand, the non-respect of the waiting period by the breeders as well as the absence of their awareness of the risks involved.

The results of the analysis revealed a high proportion of positive samples in the milk samples from the two sites hosting a dairy production organization in the Koulikoro region of Mali. Anything that makes it possible to say that the raw cow's milk produced and valued by mini-dairies contains multiple antibiotic residues to varying degrees and denotes a lack of compliance with the prescriptions related to the withdrawal period, the dose, duration and frequency of administration of antibiotics to milk-producing animals. High exposure of bacterial agents in animals (cows) to these antibiotic residues can be the cause of the emergence of resistant or multi-resistant strains on the one hand and, above all, risks to human health on the other.

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