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Prevalence of Bovine Subclinical Mastitis and Antibiotics Susceptibility Profiles of Isolates

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Abstract: The objective of this study was to recognize the bacteria involved in bovine subclinical mastitis and their resistance to selected antimicrobial agents. For this, 100 cows from 15 herds of Algerian center were randomly allocated and screened by the CMT (California Mastitis Test). The positive samples were analyzed by conventional bacteriological and Speed Mam® Color and tested for antimicrobial resistance of bacteria isolated. Bacteriological analysis shows that there is a wide range of germs that cause mastitis with 40% of *Staphylococci aurus*, 12% of *Streptococci* sp., 4% of Enterobacteriaceae, 4% of *Pseudomonas* sp., 12% for the association *Staphylococci aureus*+*Streptococci* sp., 8% for the association *Streptococci* sp.+*E. coli*, 8% for the association *Staphylococci aureus*+*E. coli*, 4% for the association *Staphylococci aureus*+*Mycoplasma* sp. and 4% for the association *Staphylococci aureus*+*Streptococci* sp.+*E. coli* (or contamination). Sensitivity tests were applied to different isolated strains and reveals the existence of resistance to antibiotics tested with varying proportions goal. However, no resistance of these organisms is noted for the danofloxacin and the association Amoxicillin+Clavulanic acid. This association of antibiotics would be the treatment of choice of the mastitis.

Key words: Milk, subclinical mastitis, CMT, bacteria, antibiotic resistance

INTRODUCTION

Mastitis being the most prevalent and the most costly disease of dairy industry worldwide with over 1.7 billion dollars a year in losses in the US alone (Sahoo *et al*., 2012), characterized by an increase in somatic cells especially, leukocytes in the milk and by the pathological changes in the mammary tissue (Ranjan *et al*., 2010).

Apart from causing colossal economic losses this disease also posses the risk for the transmission of zoonotic diseases like tuberculosis, brucellosis, leptospirosis and streptococcal sore throat to human beings (Bachaya *et al*., 2011). Various forms of clinical and sub clinical mastitis occur in bovines. In the clinical mastitis all the 5 cardinal signs of udder inflammation (redness, heat, swelling, pain and loss of milk production) are present while the sub-clinical form is bereft of any manifestation of inflammation.

The detection of mastitis is difficult. It is based on observation of symptoms by the farmer either on an indirect measure the somatic cell count in milk (Reza *et al*., 2011). In both cases detection is usually late and nothing about the identity of the organism involved.

Mastitis is considered as one of the major cause of antibiotics use in dairy animals (Sudhakar *et al*., 2009). The use of antibiotics in dairy cattle industry for the treatment and prevention of mastitis in addition to its significant economic cost (Kossaibati and Esslemont, 1997; Huijps *et al*., 2008) because very often untargeted in many cases promotes the selection of resistant bacteria (Heleili *et al*., 2012). Thus, antibiotic resistance may be a cause of treatment failure it can also result in treatment failure in other conditions, transfer of resistance genes.

Treatment failure in mastitis is due also to indiscriminate use of antibiotics without testing *in vitro* sensitivity. This practice increases economic losses thus, it is essential to identify and quantify pathogens and their resistance to assess the adequacy of the therapeutic arsenal avoid further complications, adapt and maintain efficiency on a larger scale and finally for implementation of effective control of mastitis.

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Considering this point in view, the present study was performed and aimed to estimate the prevalence of mastitis in cattle and germs implicated in these, a further objective was to determine the susceptibility of these bacteria to 6 antimicrobial agents that are or have been commonly used in dairy cows in Algeria.

MATERIALS AND METHODS

Inclusion criteria: A cow to be included in the study (taken) must present at least one quarter positive to CMT and have not received systematic or local treatment of any nature within the last 15 days.

A questionnaire containing information about the animal examined and tested is attached to every sample collection. Milk samples are collected and sent to the Regional Veterinary Laboratory of Laghouat in an insulated container and analyzed in the microbiology unit.

Study area and animals: The study was conducted in the central region of Algeria, specifically in the provinces of Blida and Ain Defla. This temperate zone is considered like a dairy basin. The weather and climate data (an average rainfall variable of approximately 500-600 mm $year^{-1}$), a series of stages from South arid climate in the valley to South wet on the reliefs give this area a character conducive to the development of breeding.

According to the agricultural services, the Blida has a total of 18,920 cattle heads with 9,500 dairy cows including 4,920 dairy cattle imported. The wilaya of Ain Defla has an estimated cattle population of 37,730 including 6,205 of dairy cows imported and 15,685 cows local and crossbred cows.

Farms characteristics: Ease of access to farms and the availability of breeders and their receptiveness to such studies are the selection criteria. A cross-sectional survey was conducted in fifteen cattle farms between May and July 2011 which correspond to the seasons of Spring and Summer during which forage availability allows an optimal milk production.

During the dry period with an average duration varies from 1-4 months, the cows in one farm are routinely subjected to an intra-mammary antibiotic after the last milking.

About 80% of the farms visited are semi-intensive. For half of these farms, milk production is not a source of income the milk is intended for home consumption. For the other half, milk is sold to the dairy.

The level of hygiene of animals and therefore livestock buildings is poor overall. Before milking, the teat washing is done. There is no teat dip or use of individual towels. Milking is manual except for a single farm with milking is done by a carriage milker and takes place in the barn.

At the time of each examination, information about breed, age, parity, stage and rank of each cow, degree of quarter attack, type of husbandry system and the village site were recorded.

Collection of milk samples and screening for mastitis: About 100 cows present within 15 farms visited are sampled individually and 396 milk samples from individual quarters (four quarters proved non-functional) are analyzed by the CMT, a qualitative measurement of the SCC in milk. It is a screening test for subclinical mastitis that can be used easily at the cowside (Bastan *et al*., 2008). The principle of the test is that when detergent is added into milk sample it causes rupture of somatic cell and release DNA and other cell contents. DNA is acid in nature while detergent contains alkyl-aryl-sulfonate which is basic in nature. DNA and detergents unite to form a gel, consistency of gel depends upon the number of somatic cells. More cells more thick gel and vice versa (Bosse, 1982; Fadrig, 1988; Oaki, 1990). The change in consistency of milk indicated mastitis while no change in consistency of milk indicated healthy samples. The mastitis was graded into categories based on the severity of disease from lower to higher intensity. The CMT reaction was graded from 0-4.

Laboratory analyzes: The positive samples to CMT were subject to a bacteriological study to identify pathogens. Two methods of bacteriological analysis are used: Speed® Color Mom and classical bacteriology. The Mom Speed® Color allows an identification of germs that cause mastitis and offers sensitivity (there is or no an antimicrobial resistance of this germs to an antibiotic).

Culture and identification of microorganisms: The samples were inoculated on nutrient agar and blood agar and Chapman's agar. The plates and broths were incubated under aerobic conditions at 37°C for 18-24 h.

Identification of the isolates was done on the basis of colony morphology, microscopic examination of Gram stained smears (Gram's staining) and catalase production Gram-negative species were identified by the API 20E (Biomerieux, Crappone France). *Staphylococcus* sp. were identified by API Staph Systems. The other strains of isolates were identified morphologically, microscopically and biochemically according to the method of Waage *et al*. (1999). A sample is considered contaminated if this contains more than two bacterial species. Isolates are subject to an antibiotic.

Antimicrobial susceptibility testing: Antimicrobial susceptibility test was performed on Speed® Mam Color Method (Manner, 2001), a micro-tray containing a specific growth medium including 14 wells testing the efficiency of 14 various antibiotics (or association of antibiotics). The susceptibility testing against the pathogenic germs is obtained within 24 h.

RESULTS AND DISCUSSION

CMT results: The CMT positivity rate for all the samples is illustrated by a low percentage (25%) which of cows tested have a subclinical mastitis justifying the bacteriological analyses leading effectively to isolate germs in most cases except for one cow for what the bacteriology was negative. According to the results, CMT reactions were considered indicative of mastitis.

A strong variation is observed between farms. In seven farms, no subclinical mastitis is identified, a rate of 46.66%. The rate of infected herds (at least one cow positive to CMT) is 53.33% (Table 1). These farms are all semi-intensive with a lack of milking hygiene.

Of 396 samples collected, 100 of them were positive to the CMT. The incidence of subclinical mastitis, averaged 25.25% in this investigation as based on udder quarters (Table 2).

The value reported in this study is similar to that obtained in France (25%) (Longo *et al*., 1994), Spain (33.5%) (Ares *et al*., 1995) and in Venezuela (30.18%) (Ferraro *et al*., 1999).

In other Maghreb countries including Morocco the frequency of subclinical mastitis was 50% (Bouaziz, 2005).

Table 1: Prevalence of subclinical mastitis in cows on farms due to screening by CMT

No. of farms visited	No. of cows tested by CMT	No. of cows positive	CMT results in number and percentage (%)
01	3	2	(2/3) (66.60)
02	9	4	(4/9) (44.40)
03	4	0	(0/4) (0.00)
04	3	1	(1/3) (33.30)
05	7	0	(0/7) (0.00)
06	11	0	(0/11) (0.00)
07	3	1	(1/3) (33.33)
08	6	0	(0/6) (0.00)
09	5	0	(0/5) (0.00)
10	4	0	0/4) (0.00)
11	3	2	(2/3) (66.60)
12	3	1	(1/3) (33.30)
13	11	0	(0/11) (0.00)
14	3	1	(1/3) (33.30)
15	25	13	(13/25) (52.00)
Total cows	100	25	(25/100) (25.00)
Total cattle	15	-	(8/15) (53.30)

Table 2: Incidence of sub clinical mastitis per quarter

No. of examined quarters	Scores of CMT test 1	2	3	4	Total	Percentage	Normal quarters
396	34	26	24	16	396	25.25	296

The difference in prevalence of sub-clinical mastitis observed in the present and the earlier studies may be due to differences in management practices use of different methods of diagnosing of sub-clinical mastitis (CMT, bacteriological examination, SCC, the Modified White Side test, pH, chlorine and catalase tests), breeds of the animals, immune response of animals, climatic conditions and the definition of infection which is variable according to the researchers (Eberhart, 1986).

Bacteria isolated during subclinical mastitis: The results of bacteriology performed on milk CMT positive samples are reported in Table 3 and 4. Indeed of the 25 positive milk samples, 15 have only one bacterial species, 8 have two, one has three (contaminated sample) and a sample has not contain any bacterial species leading to 31 isolates (Table 3).

The two bacterial species most frequently isolated were Staphylococci (40%) and Streptococci (12%). Other bacteria were isolated with variable and low frequency as shown in Table 4.

Staphylococci are the most pathogens frequently isolated from subclinical mastitis which is in agreement with Harini and Sumathi (2011). They represent almost half of the bacteria involved with a frequency of 40%. Similar results were reported by Saddek *et al*. (1996), Fallet (1999), Hassen *et al*. (2003), Tenhagen *et al*. (2006) and Bitew *et al*. (2010).

Similar results were reported in France by Bouchot *et al*. (1985) (39.0%), Longo *et al*. (1994) (44.7%) and Fabre *et al*. (1997) (29.0%) in Egypt by Saddek *et al*. (1996) (29.1%), Fallet (1999), Hassen *et al*. (2003), Tenhagen *et al*. (2006), Bitew *et al*. (2010), Harini and Sumathi (2011) and Benhamed *et al*. (2011).

Table 3: Number of bacterial species isolated by sample

No. of samples	No. of bacterial species
1	0 (4%)
15	1 (60%)
8	2 (32%)
1	3 (4%)

Table 4: Frequency of bacterial strains isolation from subclinical Mastitis.

Organisms isolated	No. of isolates	Percentage
Staphylococcus aureus	10	40
Streptococcus sp.	3	12
Enterobacteriaceae	1	4
Pseudomonas sp.	1	4
Staphylococcus aureus+*Streptococcus* sp.	3	12
Staphylococcus aureus+*Mycoplasma* sp.	1	4
Streptocoque sp.+*E. coli*	2	8
Staphylocoque aurues+*E. coli*	2	8
Streptococcus sp.+*Staphylococcus aureus* +*E. coli* (contamination)	1	4
Sterile collection	1	4
Total	25	100

However, lower frequencies are reported by Lafi *et al.* (1994) (12%) in Jordan, 16% by Busato *et al.* (2000) in Switzerland. The results obtained show that this so-called milking mastitis (major pathogens in mammary tank) predominates. Indeed, almost 60% of bacterial isolates show staphylococci or streptococci. This is due in the opinion to the lack of enforcement of basic rules to fight against mastitis proper hygiene of the milking teat dips, etc. The majority of the farms visited practiced hand milking whose hands are the main vector of organism causing mastitis (Myllys *et al.*, 1994).

Researchers also note that the third category of germs highlighted is that *Pseudomonas* sp. which represent 4% of bacterial isolates which is a lot for a group of pathogenic germs deemed minor. This result is similar to those of Heleili *et al.* (2012) who reported an infection rate with Pseudomonas of 3.03%.

The increasing prevalence of the target bacteria in mastitis can be explained in the opinion by the lack of pre-teat dips during the preparation of the udder to milking as well as a post-teat dips misbehaved or absent in late trading favoring the transmission of these pathogens to a healthy udder. Results which are similar to the result are obtained in other countries.

A survey conducted in the Netherlands highlights the importance of contagious mastitis staphylococcal and streptococcal (Miltenburg *et al.*, 1996). The results of another study conducted in Ontario tend to be closer to ours with a very significant isolation of staphylococci representing almost 40% of isolates from mastitis milk (Sargeant *et al.*, 1998).

In Sudan, it would be of interest to note that the organisms isolated in clinical and subclinical mastitis in Zebu cattle were *Staph* sp. 50 and 68.7%. *Strept* sp. 15.3 and 12.6%, coliforms 11 and 4.8% (Bagadi, 1970).

In Jordan, it was reported that the most common organisms isolated from clinical and subclinical cases were *Staph* sp. 30% (Lafi *et al.*, 1994).

Analysis conditions: The method chosen for susceptibility testing is the Speed® Color Mom. There are other methods but it has several advantages it is fast the specific susceptibility of the pathogen was obtained in 24 and 48 h in bacterial identification, reliable (performance compared to the classical bacteriology) with a sensitivity of 92% and a specificity of 96% (Manner, 2001) and many antibiotics can be tested simultaneously. Mom Speed® Color is used to isolate and identify the causative agents of mastitis. During a treatment failure or relapse, Mom Speed® Color enables a precise diagnosis and to determine the profile of antibiotic resistance.

Sterile sample: The results of bacteriology have 4% (24 positive samples following a bacteriology of 25 samples positive CMT) of results sterile. These results are far from those found by Miltenburg *et al.* (1996) (27.4%) or Fabre *et al.* (1997) (31%) or Sargeant *et al.* (1998) (17.6%).

The absence of bacterial culture despite a positive CMT can be explained by several ways. The CMT test identifies correctly 75-78% of infected quarters (sensitivity) by a major contagious pathogen or environmental origin and not 100% (Sargeant *et al.*, 2001; Dingwell *et al.*, 2003). In this case the CMT is positive but the bacteriology analysis is negative. It may be an inflammation of the udder without infection which is rare and then the sample is truly sterile.

Although, the etiology is infectious, sampling can be sterile the first possibility is the presence of antibiotics in milk that prevent germs from growing. In the study, all samples are carried out on animals that have undergone no antibiotic treatment as reported by farmers and veterinarians. The presence of infectious mastitis in which the milk is actually sterile when collected (because the germ has been removed of course) can not be ruled out (Eberhart *et al.*, 1979) as well as that of mastitis caused by germs other than bacteria (example: mycotic mastitis). Indeed, mastitis is associated in 90% of cases in the presence of bacteria. Causes fungal, viral and traumatic share the rest of the cases (Tadich *et al.*, 1998).

In the opinion, the presence of a sterile collection might be the results of false positive test CMT that is to say related to the presence of somatic cells other than inflammatory cells (especially neutrophils) and this is the case in the study of an aged cow in early lactation has not been well prepared at drying off. These cells reacted with the active test CMT which gave us a positive response despite a lack of inflammation of infectious origin. The presence of antibiotic residues may explain falsely negative bacteriological results (Longo *et al.*, 1994) because the withdrawal time is not respected in the herds.

Samples contaminated: In the study, only 4% (1 of 25 positive samples contains three germs in combination) of samples are found to be contaminated that is to say containing more than two bacterial species. In other studies, the samples are contaminated from 3-8.3% of samples (Noireterre, 2006). In the opinion, the low percentage of samples contaminated sign a good command of gesture and a good udder preparation before sampling.

Resistance to antibiotics in bacteria isolated from subclinical mastitis: Antibiotic resistance of bacteria isolated from subclinical mastitis is weak although, there are some exceptions (Table 5 and 6).

Table 5: The global susceptibility of Gram+cocci strains isolated from subclinical mastitis to some antibiotics

Antibiotics	Number N	Resistant (%)	Susceptible (%)
Amoxicillin+Clavulanic acid	13	0.00	100.0
Ampicillin+Colistin	13	10.00	90.00
Cefalexin	13	10.00	90.00
Cefoperazon	13	16.67	83.33
Cefquinon	13	10.00	90.00
Cloxacilin	13	68.33	31.67
Danofloxacin	13	0.00	100.0
Gentamicin	13	26.67	73.33
Marbofloxacin	13	05.00	95.00
Penicillin G+Streptomycin	13	38.33	61.67
Spiramycine	13	68.33	31.67
Sulfadimidin+Trimetoprim	13	80.00	20.00
Tetracycline+Neomycin+Bacitracin	13	43.33	56.67
Tylosin	13	63.33	36.67

Table 6: Antimicrobial sensitivity tests of staphylococci, streptococci and enterobacteria isolated from subclinical mastitis

	Germs involved		
Antibiotics	*S. aureus*	*Streptococcus* sp.	Enterobacteriaceae
Amoxicilin+Ac. clavulanic	+++	+++	+++
Ampicillin+Colistin	++	---	+++
Cefalexin	++	---	+++
Cefoperazon	+++	++	+++
Cefquinon	++	+++	+++
Cloxacilin	---	---	+++
Danofloxacin	+++	+++	---
Gentamicin	++	++	+++
Marbofloxacin	++	+++	+++
Penicillin G+Streptomycin	++	---	---
Spiramycin	---	---	---
Sulfadimidin+Trimetoprim	---	---	---
Tetracycline+Neomycin+ Bacitracin	++	---	+++
Tylosin	---	---	---

+++ = Sensitive, ++ = Intermediate, --- = Resistant

The global susceptibility of bacterial isolates strains showed a susceptibility of 100% for the association Amoxicillin+Ac. Clavulanic and Danofloxacin or nearly 100% for Marbofloxacin, Cefquinon, Cefalexin and the association Ampicillin+Colistin. On the contrary, antibiotics showing higher rate of resistance patterns were the association Sulfadimidin+Trimetoprim, Cloxacilin, Spiramycine and Tylosin with 80, 68, 33, 68, 33, 63 and 33%, respectively.

The global susceptibility of Gram+cocci isolates has revealed that 38.33% of the strains are resistant to the association Penicillin G+Streptomycin, 26.67% to Gentamicin and 43.33% to the association Tetracyclin+ Neomycin+Bacitracin.

No resistance was showed against the association Amoxicillin+Clavulanic acid, Danofloxacin but some resistance has been noted against Cefoperazon (16. 67%). The results of antibiotic resistance in bacteria isolated have much in common with those reported in the literature showing the high sensitivity of staphylococci to the most of antibiotics.

The susceptibility of *Staphylococcus aureus* to the tested antibiotics has revealed a complete (100%) susceptibility to the association Amoxicillin+Clavulanic acid, Cefoperazon and Danofloxacin. The same results were reported by Heleili *et al.* (2012) for the association Amoxicillin+Clavulanic acid. However, a very high frequency of resistance of *staphylococci* to penicillin G (83.5%) was reported (Rahal, 2001). Unakal and Kaliwal (2012) reported a high rate of resistance against the Gentamycin but it is not the case in the study.

Next in a declining order, researchers have found the association Penicillin G+Streptomycin, Marbofloxacin and Cefalexin (90% each), the association Ampicillin+Colistin, Gentamicin, the association Tetracycline+Neomycin+ Bacitracin and Cefquinon (80% each). Results of Bouaziz (2005) and Heleili *et al.* (2012) confirm the findings. A rate of resistance has been shown against Cloxacilin and Spiramycin (70% each) and against the Sulfadimidin+ Trimetoprim and Tylosin (60% each). Followed the survey realized on land with veterinarians and farmers these antibiotics are the more marketed in center region of Algeria.

The high resistance of these bacteria for this type of antibiotic is linked in the opinion to the misuse of this type of antibiotic by veterinary practitioners saw its cheaper price compared to other antibiotics and its currently used to prevent or cure different animal infections which creates this strong resistance by selection of resistant strains and probably followed by transfer of resistance genes.

This suggests that the antimicrobial agents commonly used in commercial preparation are used indiscriminately and that bovine strains of *S. aureus* have probably evolved into resistance. This result is in contradiction with those obtained by Heleili *et al.* (2012) in Eastern region of Algeria for these antibiotics. This difference of resistance for this antibiotic can due to the difference of use of antibiotic that differed of area at other. Antibiotic resistance varies also according to country: in Tunisia, high frequencies of resistance to penicillin G (60%) were observed by Hassen *et al.* (2003).

The high resistance of streptococci (100%) obtained in this study with respect to the association: Sulphadimidin+Trimetoprim is well above the rate of 64.60 and 35%, respectively reported by Seleim *et al.* (2002), Messadi *et al.* (1999) and Bouaziz (2005).

The high strength (67%) of streptococci with respect to the Tetracycline is comparable to those obtained by Messadi *et al.* (1999) who reported a frequency of tetracycline resistance by 64%. However, it is above of the rate reported by Makovec and Ruegg (2003) (54%) or by Bouchot *et al.* (1985) (30%) and Bouaziz (2005) (40%).

Streptococci were sensitive to Cephalosporin. Indeed, low or no resistance has been demonstrated for cephalosporin. This result is in agreement with those of several researchers (Owens *et al*., 1997; Myllys *et al*., 1998; Bouveron, 2001). These researchers reported low frequencies of resistance of this organism with respect to Cephalosporin.

The resistance of streptococci to the association containing Tetracycline is high 67% of streptococci were resistant. The results deviate from those reported in the literature from 0-27% (Bouveron, 2001).

The global sensitivity of Gram-negative bacilli has revealed a high degree of resistance against Danofloxacin, Penicillin G+Streptomycin, Sulfadimidin+Trimetoprim and Tylosin (100 each %). Nevertheless, Gram-negative bacilli showed a complete susceptibility to most antibiotics (Table 5). Similar results werere ported by Bouchot *et al*. (1985) and Heleili *et al*. (2012).

The present study has revealed that most of the strains isolates showed a high sensitivity against the antibiotics tested but some resistances were obtained for others antibiotics, largely used on land. This proves that there is an indiscriminate use of these types of antibiotics in animals in the center region of Algeria.

The resistance observed for some antibiotic can be explained by the fact that these antibiotics are used as a food additive.

Finally, researchers must remember the difficulty of comparing the results with those of other researchers because the techniques for measuring antibiotic sensitivity are different (seeding method, technique disks, technical agar dilution, technical micro-broth dilution) as the interpretation criteria are different from one study to another (Gentilini *et al*., 2000; Erskine *et al*., 2002). However, in the study all bacteria showed sensitivity for danofloxacin and the association amoxicillin+Clavulanic acid which is an interesting choice for veterinarians wanting to avoid the problem of antibiotic resistance. For the part, the sensitivity of bacterial strains to these antibiotics is attributed to its non-use by veterinary practitioners and employment in the most cases other antibiotics (Tetracycline, Colistin, Gentamicin and Ampicillin). This was demonstrated in a survey of veterinary practitioners.

CONCLUSION

Like this study, researchers can enumerate some points:

- Mastitis is a dominant disease in dairy cattle farms in the central region of Algeria with a prevalence of 25%
- The high resistance of pathogens isolated from bovine mastitis for some antibiotic is probably a reflection of both transmissibility of resistant organisms and bad practices of antibiotic use in the industry (misuse of antibiotics)
- The high frequency of resistance toward these antibiotics are widely used in veterinary medicine could explain the treatment failures encountered in the field
- The evolution of mammary infections in livestock requires regular bacteriological monitoring to adapt the prophylactic and treatment plan. However, efforts must be maintained to establish a follow-up of these resistances as well as consumption and conditions of use of antibiotics in the treatment of bovine mastitis

The use of antibiotics can lead to resistance. To minimize this risk, researchers must observe the following precepts: use antibiotics in a focused and as seldom as possible. Do not use antibiotics to correct errors of hygiene and management. Follow directions of the veterinarian.

The caution in the use of antibiotics if we still want to benefit in the future. An abusive administration always results in inefficiency in the short or medium term. Finally, application of good sanitary and hygienic measures.

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