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Evaluation of the resistance to antibiotics of bacteria responsible for mastitis in cows in Southern Algeria

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Abstract

The aim of this study is to evaluate the sensitivity of germs isolated from mastitis towards different antibiotics.The work carried out on several cattle herds in the South region of Algeria. A total of 80bacterial strains were isolated to be the subject of a disk-diffusion agar antibiotic sensitivitytest,the bacterial strains were under the groups of Staphylococcus coagulase positive SCP (10), Staphylococcus coagulase negative SCN (35) andEnterobacteriaceae (35).Twenty-one antibiotic's effectiveness were put to the test. The results showed that 100% of the SCP strains were resistant to Penicillin G, while the SCN strains were less resistant to it with a resistance rate of71.42%. SCN strains' resistance was high towards oxacillin (68.57%), erythromycin (54.28%), clindamycin (54.28%), and for vancomycin (51.42%). However,no resistance was recorded against gentamycin. Enterobacteriaceae strains had a high resistance against the combination: Amoxicillin + clavulanate(74.28%).

On light of the results, the following antibiotics:Gentamicin, the combination Trimethoprim-sulfamethoxazole and enrofloxacin tend to be the best antibiotics to fight against mastitis in the center region of Algeria.

It must be noted that while being very effective, it's not recommended to use Gentamicin, because it is not that available in our region and is very costly; on the other hand we recommend the use of Sulfamides due to their availability in the Algerian market and being cheaper.

Key words: Mastitis, antibiotics, resistance, Southern Algeria.

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1. Introduction

Globally, Bovine mastitis is the main cause for which antibiotics are used in farms [Erskine, 2000]. Several pathogens have been isolated after the analysis of samples taken from mastitis cases (Bradley ET Green 2000).

In dairy herds the number one measure used to prevent and treat mastitis is antibiotic based therapy (Erskine, 2000). However, and even with long term treatment using antibiotics in some cases mastitis still prevails. Moreover, there are increasing concernsregarding the use of antibiotics in animal feed and its role in the transmission of resistant bacteria or resistance genetic determinants to human pathogens through the food chain; these concerns state that the use of antibiotics in animals feedhas influence with resistant pathogens in animal source food like milk. This hypothesis however is yet to be confirmed, it has to be clinically verified within the environment of a dairy farm, because unfortunately here in Algeria, within the dairy industry there is little to no data on this subject. Because of that and due to this situation, it was necessaryto obtain the needed information and to prove the existence of relation between the use of antibiotics and the resistance of the mastitis causing bacteria.

With the Algerian animal husbandry's condition in mind several questions need to be asked among them: what is the rate of resistant bacteria that cause mastitis within a dairy herd, and what is the size of their resistance spectrum towards the antibiotics available in the Algerian market?

To answer the previously asked questions, here we present a study with the aim to determine the profiles of the antibiotic resistance in bovine mastitis causing bacteria.

To confirm whether or not the bacteria causing mastitis within a herd are resistant to the antibiotics used in the farm we executed an antibiotic resistance profiling which will determine whetherthe bacteria are resistant or sensitive to the different antibiotics used in the farm.

1. Material and Methods

1.1. Bacterial strains

For this study, several bacterial strains were isolated from clinical and subclinical mastitis cases, with the aim to evaluate invitro their sensitivity towards antibiotics, the strains were isolated in 2012 and the study took place in the regional laboratory of Tlemcen.

There were 80 strains and they fall under the following groups: Staphylococcus coagulase positive SCP (10), Staphylococcus coagulase negative SCN (35) and Enterobacteriaceae (35).

1.2. Antibiotics

Table 1 represents a list of the tested antibiotics with their respective charges

1.3. Sensitivity test

The test was carried out in-vitro and it followed the disk-diffusion agar method andKirby Bauer technic of the antibiogram committee of the French microbiology society [CA-SFM] [Zadoks ET AL, 2003]. Muller-Hinton [Bio-rad] was the medium used in the test. The Muller-Hinton was uniformly disposed in a petri dish with a thickness of 4 mm (25ml for the dishes with a diameter of 9cm). Before their use the dishes must be dried for 30min in 37C°

The test was carried out in the following manner:

1.4. Inoculum

- Using a swab, sample few identical well insulated colonies from a pure culture grown for 18hours on an isolation medium.

- Exonerate the samples of the swab's cove in 10ml of a sterile saline solution.
- Mix the bacterial solution until it becomes homogenous with an opacity of 0.5 unity of McFarland standards.
- Adjust by adding more samples if it's soft or the saline solution if it's too dense.
- The Culturing should start within the 15 min following the preparation of the inoculum.

Table 1: Antibiotics used in the present study

Antibiotics	Code	Charge
β LACTAMS		
Penicillin G	P	6µg
Ampicillin	AMP	10µg
Amoxicillin	AM	30µg
Oxacillin	OX	1µg
Amoxicillin + clavulanate	AMC	10µg
Cefoxitin	FOX	30µg
Ceftiofur	XNL	30µg
Cefotaxime	CTX	30µg
CEPHALOSPORINES		
Cefodizime	Cz	30µg
AMINOGLYCOSIDES		
Clindamycin	CM	2µg
Gentamicin	G	10µg
Neomycin	N	30µg
MACROLIDES		
Erythromycin	E	15UI
LINCOSAMIDES		
Vancomycin	VA	30µg
SULFAMIDES -TRIMETHOPRIMES COMBINATION		
Trimethoprim Sulfamethoxazole	SXT	1,25 – 23,75 µg
TETRACYCLINES		
Tetracycline	TE	30µg
FLUOROQUINOLONES		
Enrofloxacin	ENR	5µg
QUINOLONES		
Flumequine	FT	30µg
Nalidixic Acid	NA	30µg
PHENICOLES		
Chloramphenicol	C	30µg
POLYPEPTIDES		
Colistin	Cs	50µg

1.5. Culturing

- Soak a sterile swab in the bacterial solution.
- While taking the swab out, gently wring the swab on the tube's wall to eliminate any inoculum glut.
- Rub the swab's cove on the surface of the agar (within the dish) up and down creating close to each other diagonal strips.
- Repeat the process, but each time turn the dish 60° to create crossed strips.

- The dish must be kept fresh but with no liquid traces

1.6. Disk application

- Using a distributor, apply the [Bio-rad] antibiotic disks

1.7. Incubation

- After 15min after the inoculation the dishes are incubated for 18hours at 35C°.

1.8. Reading and interpretation

- The reading is done through measuring the diameter of the inhibited area using a caliper.
- To classify weather a bacterial strain is susceptible (S), intermediate (T) or resistant (R) to the antibiotics presented by the disks, we compare the diameters of the inhibited areas obtained with the critical diameter published by well-known organizations.
- In our test the interpretation is done according to [Bio-rad] indications.
- The strains with an inhibition area diameter smaller than the resistance threshold are considered resistant, the strains with a diameter bigger than the susceptibility threshold are considered susceptible, and those with a diameter between the two thresholds are considered intermediate.

1.9. Data Analysis

Microsoft Excel 2003 was used for the analysis and treatment of data. This software has given us the possibility to represent through figures the rates of different variables.

We wanted to use a statistics software to compare the results of different species, however due to difference in the number of these species were unable to do the comparison and complete the statistical analysis.

2. Results

2.1. Staphylococcus coagulase positive

The SCP strains'susceptibilityto the 12 tested antibiotics is shown in table 2

100% of the SCP strains were resistant to the penicillin G, 50% were resistant to the oxacillin, 40% had resistance towards tetracycline, for erythromycin, clindamycin, vancomycin, enrofloxacin, cefoxitin and the combination amoxicillin + clavulanate the resistance rate was 10% and 30%. No resistance was show against the remaining antibiotics (gentamycin & neomycin) meaning that from the 12 tested antibiotics only gentamycin &neomycin were 100% effective against all SCP strains.

The antibiogram we ran on the SCP strains showed that 11 out of the 12 tested antibiotics had and effectiveness that was equal and exceeded 50%, and the Penicillin G was the antibiotic with the least effectiveness on the strains.

2.2. Staphylococcus coagulaseNegative

The SCN strains' susceptibility to the tested antibiotics is shown in table 3

Compared to the SCPs the SCN strains were less resistant to penicillin G with a resistance rate of 71,42%. SCN strains show a high resistance towards oxacillin (68,57%), erythromycin (54,28%), clindamycin (54,28%), and towards vancomycin (51,42%). The SCN strains were susceptible to neomycin, enrofloxacin and the combination Trimethoprim + Sulfamethoxazole with a susceptibility rate 80% however, these strains were completely susceptible against gentamycin. Cefotaxime and Tetracycline had an effectiveness rate of 54,28% and 65,71% respectively.

Table 2: SCP's susceptibility towards 12 antibiotics

Antibiotics	Total	n= 10					
	Profile	Susceptible		Resistant		Intermediate	
	Break points	Number	%	Number	%	Number	%
P	≤ 28-29≥	0	0	10	100	0	0
OX	≤10-13 ≥	5	50	5	50	0	0
FOX	≤19-20≥	8	80	2	20	0	0
AMC	≤ 19-20≥	8	80	2	20	0	0
ENR	≤16-23≥	9	90	1	10	0	0
VA	≥15	8	80	2	20	0	0
SXT	≤10-16 ≥	9	90	0	0	1	10
CM	≤14-17≥	7	70	3	30	0	0
GM	≤12-15≥	10	100	0	0	0	0
TE	≤14-19 ≥	6	60	4	40	0	0
N	≤ 13-18≥	10	100	0	0	0	0
E	≤ 13-23≥	7	70	3	30	0	0

Table 3: SCN's susceptibility towards 12 antibiotics

Antibiotics	Total	n= 35					
	Profile	Susceptibility		Résistance		Intermediate	
	Break points	Number	%	Number	%	Number	%
P	≤ 28-29≥	10	28,57	25	71,42	0	0
OX	≤17-18 ≥	5	14,28	24	68,57	6	17,14
FOX	≤24-25≥	19	54,28	11	31,42	7	20
AMC	≤ 19-20≥	18	51,42	17	48,57	0	0
ENR	≤16-23≥	30	85,71	1	02,85	4	11,42
VA	≥15	17	48,57	18	51,42	0	0
SXT	≤10-16 ≥	32	91,42	1	02,85	2	05,71
CM	≤14-17≥	16	45,71	19	54,28	0	0
GM	≤12-15≥	35	100	0	0	0	0
TE	≤14-19 ≥	23	65,71	12	34,28	0	0
N	≤ 13-18≥	34	97,14	1	02,85	0	0
E	≤ 13-23≥	10	28,57	19	54,28	6	17,14

2.3. Enterobacteriaceae

The Enterobacteriaceae strains' susceptibility to the tested antibiotics is shown in table 4.

The resistance and susceptibility of the Enterobacteriaceae strains towards the 16 tested antibiotics is shown in table 4.

The strains showed weak resistance against nalidixic, Chloramphenicol and colistin with a rate of (5,71%) as well as against the combination Trimethoprim + Sulfamethoxazole (2,85%)

This rate increases to20% for tetracycline and 28,57% for amoxicillin. The Enterobacteriaceae strains show a high resistance towards the combination amoxicillin + clavulanate (75,28%) and a lower resistance rate against the β lactamases.

The effectiveness of every antibiotic tested on the isolated bacteria is shown in table 5.

The summary of the of the effect of the different antibiotics on the bacterial strains SCP, SCN and Enterobacteriaceae shows that the aminoglycosides, the fluoroquinolones and the combination of Trimethoprim & Sulfamethoxazole are the most efficient antibiotics with an effectiveness rate that exceeded 80% against the groups of the isolated bacteria. The summary also shows that on the other hand the bacterial groups were somewhat resistant against the tetracyclines and the β lactamases with a resistance rate exceeding 50%.

Table 4: Enterobacteriaceae's susceptibility profile towards 16 antibiotics.

Antibiotics	Total	n= 35					
	Profile	Susceptible		Resistance		Intermediate	
	Break points	Number	%	Number	%	Number	%
CTX	≤14-23 ≥	29	82,85	0	0	6	17.14
AMC	≤ 13-18≥	8	22,85	26	74,28	1	02,85
FOX	≤14-18 ≥	29	82,85	5	14,28	1	02,85
SXT	≤10-16 ≥	34	97,14	1	02,85	0	0
N	≤13-18 ≥	35	100	0	0	0	0
TE	≤14-19 ≥	28	80	7	20	0	0
XNL	≤17-21≥	32	91,42	0	0	3	08,57
AM	≤13-17 ≥	21	60	10	28,57	4	11,42
CZ	≤14-18 ≥	28	82,35	5	14,7	1	2.9
AMX	≤14-21≥	21	60	10	28,57	4	11,42
G	≤12-15≥	35	100	0	0	0	0
FT	≤21-25≥	30	85,71	3	08,57	2	05,71
ENR	≤16-21≥	32	91,42	0	0	3	08,57
NA	≤13-19≥	31	88,57	2	05,71	2	05,71
C	≤12-18 ≥	33	94,28	2	05,71	0	0
CS	≤10-13≥	33	94,28	2	05,71	0	0

Table 5: The effectiveness of the antibiotics on the isolated bacterial strains

	Bacteria	SCP			SCN			Enterobacteriaceae		
	Profile	**Ss [%]**	**R [%]**	**I [%]**	**Ss [%]**	**R [%]**	**I [%]**	**Ss [%]**	**R [%]**	**I [%]**
antibiotics	β Lactamases	52,5	47,5	0	38,57	53,57	07,85	66,67	24,28	09,04
	Tetracyclines	60	40	0	65,70	34,30	0	0	80	20
	Trimethoprim + Sulfonamides	90	0	10	91,42	02,85	05,71	80	20	0
	Macrolides	70	30	0	28,57	54.28	17,14	-	-	-
	Polypeptides	-	-	-	-	-	-	94,28	05,71	0
	Aminoglycosides	90	10	0	80,95	19,05	0	100	0	0
	Quinolones	-	-	-	-	-	-	85,71	05,71	08,57
	Fluoroquinolones	90	10	0	85,71	02,85	11,42	91,42	0	08,57
	Lincosamides	80	20	0	48,57	51,42	0	-	-	-
	Cephalosporines	-	-	-	-	-	-	82,33	14,70	02,94
	Phenicols	-	-	-	-	-	-	_94,28	05,71	0

3. Discussion

We must note that the comparison of the results of our study with other studies is quite difficult due to difference in the technics used to measure the resistance against the antibiotics (the culturing method, the disk technic, the medium, and the dilution in the liquid medium) as well as the interpretation criteria (Gentilini et al.2000).

Our study on the susceptibility of SCP strains towards the antibiotics has revealed a high resistance rate against the antibiotic penicillin G that reaches 100%, this rate is close to the one obtained by RAHAL (2001) 83,5%, however our rate was far superior to the rate obtained by HELEILI (2002) 18%.

The resistance rates against penicillin obtained by our study were different from the rates found by other researches from other countries.

On one hand, MARKOVIC & RUEGG (2003) reported a resistance rate of 35,5% and on another hand MYLLYS et al. (1998) reported a superior rate of 50,7%, and that's not all in Tunisia BEN HASSEN & al. (2003) obtained a 60% resistance rate towards penicillin.

The results obtained by our study concerning the resistance of Staphylococcus species towards penicillin falls into the interval of 5-90% obtained by comparative studies ran in multiple countries (De Oliveira et al. 2000). This SCP's resistance against penicillin explains S. *aureus'* ability to hyperproduceβ Lactamase.

The resistance rate of SCP strains against tetracycline obtained by our study was 40% which was close to the rate obtained by BEN HASSEN & al in Tunisia 36% (Ben Hassen et al.2003).

The wide range abusive use of Penicillin G over a long period of time has rendered this antibiotic completely useless and with no activity, Penicillin G was used as a treatment and a prevention measure against all sorts of infections without any prior analysis or antibiogram to identify the germ itself, it's causes or it's susceptibility profile, and from our point of view this led to the selection of strains with 100% resistance to the antibiotic.

Gentamycin and Neomycin recorded an effectiveness rate of 100% against SCP strains, this susceptibility in our opinion is because they were used only occasionally due to their costliness and rarity when compared to other antibiotics at hand.

Despite the red flag raised by many studies all around the globe on the resistance of various bacteria against Penicillin,

some argentines (Gentilini et al. 2000) and American (Watts et al.1995) authors have revealed from multiple studies on cattle that the proportion of resistant S. *aureus* isolated had declined between the year 1996 and 2000. These encouraging results means that a more rational and targeted use of penicillin could one day resuscitate it's dead effectiveness against these bacteria

The resistance shown by the SCN strains in our study was very different from that shown by SCP strains against Penicillin G however it was practically the same against Tetracycline.

71,42% Of the strains were resistant to Penicillin G which was quite far from the rate found by MARKOVIC and RUEGG (2003) (32,6%) for the same antibiotic.

The resistance rate towards tetracycline obtained by our study was 34,28% and it was once again superior to the rate obtained by MARKOVIC & RUEGG (2003) (22,6%)

In our study SCN strains show greater resistance towards Oxacillin, erythromycin, clindamycin and vancomycin when compared to SCP strains, this counter edict the results obtained by BEN HASSEN et al. (2003) where SCN strains were more susceptible than SCP strains against those antibiotics.

In our study, the SCN strains resistance rate towards oxacillin was 68,57% which is superior to the rates HELEILI (2002) and MESSADI et al. (2002) have obtained (34% et 36% respectively).

The resistance rate towards Penicillin G was in consonant with what MYLLYS et al. (1998) found.

The non-controlled abusive use of the antibiotics without giving much attention into the antibiogram or the analysis of the nature of the germs causing the diseases is once again the reason for which the resistance of the SCN strains isolated from mastitis cases in our local farms is very high, the antibiogram and the analysis are two essentials in order for the antibiotherapy to succeed.

The susceptibility and resistance rates of SCN strains are very close to those of the S. *aureus* with few differences.

Finnish study done by MYLLYS et al. (1998) on cattle revealed a multiple drug resistant SCN strains toward Penicillin and Erythromycin. Our study however revealed that the SCN strains are resistant to penicillin however they are susceptible against erythromycin and the combination Trimethoprim + Sulfamethoxazole.

The number one spot for inefficient antibiotic is no longer held by The Colistin as it loses it to penicillin with its effectiveness rate that is below 30%.

Our results show concerning SCN strains were quite similar to the results concerning S. *aureus* however we note that the total of SCN strains' susceptibility rate has decreases, this decrease comes from the fact that multiple Staphylococcus species form the SCN strains and the susceptibility varies from one species to another.

The Enterobacteriaceae strains show a resistance rate of 28,57% against the broad-spectrum antibiotic ampicillin. Different studies show a spectrum of various rates that goes from 13 to 95%. The rate obtained in our study is way below the rate obtained by RAHAL's study [2001] which was 85,5% however in his study the animals from which the Enterobacteriaceae strains were samples are not defined.

The Enterobacteriaceae strains' resistance rate towards tetracycline was 20% which was close to the rate obtained by MARKOVEC and RUEGG (2003) 37%, but it was superior to the 15% obtained by LEHTOLAINEN et al. (2003). This rate was also inferior to the one obtained by MESSADI & al. [2002] in Tunisia which was a 50% Tetracycline resistance rate.

Despite being known as an effective antibiotic against Enterobacteriaceaestrains colistin shows an effectiveness rate of 5,71% in our study. This antibiotic diffuses poorly in the medium (Bouaziz 2005).

In Tunisia and in a study by MESSADI et al. (2002)colistin shows an effectiveness rate of 46%. Also, in the same study done by MESSADI et al. (2002) the Enterobacteriaceae strains showed a resistance rate of 19% towards the combination Trimethoprim + Sulfamethoxazole where in our study the strains showed a very low rate of 2,85% which was close to the rates 04% & de 03,8% obtained by LEHTOLAINEN et al. (2003) et MARKOVEC and RUEGG (2003) respectively.

The Enterobacteriaceae strains in our study were highly susceptible toward cefodizime with an effectiveness rate of

82,35%, and these results are consented to the results obtained byBouchot et al. (1985), MESSADI et al. (2002) and de LEHTOLAINEN et al. (2003).

By studying the susceptibility and resistance of bacteria towards the tested antibiotic groups we were able to show that aminoglycosides, fluoroquinolones and the combination Trimethoprim + Sulfonamides are by far the most effective antibiotics.

With 100% effectiveness rate against SCP strains Gentamicin cements his place as the drug of choice against those species, these results are consent to what DE OLIVEIRA et al. (2000) obtained, qualifying Gentamicin as un excellent antibiotic against Staphylococcus.

This high Aminoxides effectiveness could be due their wide target and activity spectrum.

Tetracyclines are considered an antibiotic group with good effectiveness, and it is confirmed by the results of our study which also outlines that these antibiotics are effective against bacilli and cocci bacteria.

β Lactamases show a weak effectiveness, and this is translated by the very high resistance presented by the isolated bacteria against penicillin; which brings down the higher effectiveness shown by other drugs in the group.

On the other hand, showing the effectiveness rates of 94,28%, and 91,42 % respectively Polypeptides et Fluoroquinolones, are the most effective antibiotic groups against Enterobacteriaceaespecies.

The high susceptibility of Enterobacteriaceae strains against Polypeptides [Colistin] pays a direct homage to the drug's wide spectrum of activity. However, colistin is only active against few Gram-negative bacteria.

And despite having a wide activity spectrum, the first generation of Fluoroquinolones like enrofloxacin are only active on gram-negative bacteria, which explains why in our study the Enterobacteriaceae susceptibility was high against enrofloxacin and Colistin.

At the end it's very important to note that a high in-vitro susceptibility doesn't guarantee the recovery in-vivo, in fact when it comes to mastitis caused byStaphylococcus the in-vivo healing rate is ranging from 60 to 70%(Bouchot et al. 1985) and according to the same study this inconsistency is due to the fact that these bacteria locate themselves in the intercellular spaces, making them most of the time encapsulated and protected from the antibiotics.

SEARS et al. (1990); WILESMITH et FRANCIS (1986), show in their study that S. *aureus* beside their tendency to live inside the cells, when in-vivo it adopts another tendency, and it is to take cover within the micro abscess within the living tissues of the mammary gland making the healing and recovery process for the mastitis cause by S. *aureus* even harder.

Conclusion

The antibiogram applied in our study shows the following results: From the tested 21 antibiotics Gentamicin seems to be the most effective antibiotic, weather against Enterobacteriaceae or Staphylococcus, with an effectiveness rate that reaches 100%.

For the rest of the antibiotics the susceptibility rates vary between the Staphylococcus and the Enterobacteriaceae strains.

The effectiveness of the antibiotics on Staphylococcus coagulase negative strains is similar to their action on Staphylococcus coagulase strains but we must note a lower general susceptibility rate.

In total, following the results of our study the best antibiotics in the fight against mastitis in the center region of Algeria are these four:

Gentamicin, the combination Trimethoprim + Sulfamethoxazole and enrofloxacin.

We have to note, even though it's the most effective antibiotics gentamicin is not recommended as first choice due to its costliness and rarity. We recommend the use Sulfonamides due to their availability in the market as well as their relatively low cost.

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