

# Antimicrobial susceptibility pattern to disinfectants in *Pseudomonas aeruginosa* strains isolated from dairy sheep breeds in Sardinia



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## SUMMARY

**Introduction** - *Pseudomonas aeruginosa* is still one of the leading causes of morbidity in dairy animals and particularly associated with severe clinical forms of mastitis. Its outstanding capacity to evade the activity of antimicrobial prophylaxis makes this species one of the most feared pathogens. In this context the World Health Organization (WHO) has reported this pathogen in a catalogue of bacteria that pose the greatest threat to human health. Thus, an accurate disinfection procedure is needed to avoid the incidence of *Pseudomonas*- related diseases in veterinary as well as in human medicine.

**Aim** - The aim of this study was to evaluate the antimicrobial resistance patterns to common disinfectants in *P. aeruginosa* strains isolated from ovine breeding farms in Italy.

**Materials and methods** - In this study a set of 44 clinical *P. aeruginosa* strains isolated from 11 different sheep breeding farms with clinical cases of severe mastitis were analyzed and evaluated for susceptibility to 4 different disinfectants: Benzalkonium Chloride (BZC), Chlorhexidine (CHX), Sodium Hypochlorite (NaClO) and Hydrogen Peroxide (H<sub>2</sub>O<sub>2</sub>). The capacity of these disinfectants to inhibit *P. aeruginosa* was evaluated by using standard broth microdilution methods.

**Results and discussion** - The results showed that CHX and NaClO were the most active against this bacterium, but a great number of multi-disinfectant resistant strains were observed altogether, especially if we consider BZC and H<sub>2</sub>O<sub>2</sub>, with 55% of drug resistant strains. Moreover, a correlation was evaluated between H<sub>2</sub>O<sub>2</sub> MIC values and: (i) percentage of mucoid strains, (ii) increase in resistance patterns to other disinfectants (Pearson's  $r$  was 0.029 for CHX).

**Conclusion** - The work suggests the crucial role of disinfection in veterinary medicine and raises concern about the possibility of an inadequate prophylaxis through the use of Hydrogen Peroxide on breeding farms contaminated with *Pseudomonas spp.*

## KEY WORDS

*Pseudomonas aeruginosa*, sheep, mastitis, disinfectants, MICs.

## INTRODUCTION

*Pseudomonas* are ubiquitous Gram-negative bacteria isolated from humans, animals and the environment. Due to its adaptability it is a frequent contaminant of water pipes in several nosocomial areas as well as on animal breeding farms. This aspect is strengthened by the extremely simple nutritional requirements required for these microorganisms, for example they have been observed growing in pure distilled water<sup>1</sup>. "Pseudomonas-induced biofilm" is a concern for high economic loss in industrial water systems and for the significant sanitary impact in the veterinary field, as well as being a nosocomial pathogen in hospitals. In this context, *P. aeruginosa* is a bacterium of considerable medical importance for its high profile of pathogenicity and adaptability in various environmental conditions. In fact, acute infection by

this opportunistic pathogen is the leading cause of significant morbidity and mortality in infected subjects. In humans with impaired immune systems, this bacterium causes severe infections such as: cystic fibrosis, infections of the cornea or skin burns, catheter-related infections and lung infections in patients with emphysema and obstructive lung disease<sup>2</sup>. It is also a causal agent in both livestock and companion animals, associated to endometritis, hemorrhagic pneumonia and mastitis<sup>3,4</sup>. On ovine farms *P. aeruginosa* (Pa) could cause mastitis with peracute and severe infection due, for example, to endotoxin production or it may be associated with subacute or chronic illnesses<sup>3</sup>. In all these situations, one of the main difficulties in clinical management lies in the extensive resistance of *Pseudomonas* to antimicrobial agents, associated with a particular recognized biofilm *status* in sanitary device contamination, as well as in persistent or chronic infection in animals<sup>3</sup>. The biofilm formation of Pa represents a dynamic and complex phenomenon which undergoes a set of phases of: (i) attachment to a surface /tissue, (ii) formation of microcolonies, (ii) maturation into macro colonies,

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(ii) de-structuration and (iv) organ dissemination progression<sup>5</sup>. These different sessile states of Pa, in comparison with planktonic liquid culture, make these bacteria highly resistant to antimicrobial agents, including the disinfectants used on dairy farms against bacteria contamination, especially in milking machine pipes and tanks. This process of biofilm formation and subsequent antimicrobial resistance indicates the most probable reason for the difficulty in the eradication of this etiological agent and calls for the enforced application of cleaning and sanitation protocols. The most serious problem on dairy farms has been the change from hand milking procedures to mechanical milking machines that has come about in the last 4 decades in developed countries and diseases linked to biofilm formation in the pipe lines on milking machines are amongst the most serious and urgent sanitary problems recognized as being associated to such changes. Disinfectants play an important role on animal farms in shed hygiene and in the disinfection of different farm devices, i.e. milking machines, milk tanks and hand disinfection for farmers, etc. However, the use of a non-compliant disinfection protocol and/or the use of an inactive disinfectant could produce an increase in chemical resistant strains with severe problems in eradication of infection<sup>6</sup>. At the moment, bibliographic data report the presence of multi-drug resistant (MDR) strains of Pa on ovine farms as well as in hospitals worldwide, especially against chlorhexidine and benzalkonium chloride<sup>7,8</sup>. In this context, we believe that a disinfectant susceptibility profile linked to Pa growth inhibition could be useful in the veterinary field in protocols against Pa infections detected on ovine farms. For this reason, the aim of this study is to reveal the antimicrobial susceptibility pattern of *P. aeruginosa* strains against the common disinfectants used on sheep farms.

## MATERIAL AND METHODS

### Sampling

The analyzed breeding groups were composed of around 4000 Sardinian ewes distributed among 11 sheep farms located in different areas in Southern Sardinia. In this context we selected 50 sheep, over a period of two years, as part of the ordinary diagnostic activity performed by Sardinian Istituto Zooprofilattico Sperimentale (IZS). Clinical and bacteriological examination confirmed a high prevalence of mastitis caused by *Pseudomonas aeruginosa* on the farms in question. On examination and palpation 43 sheep were affected by unilateral mastitis with varying degrees of alteration of the modified breast tissue, with lymph node involvement and macroscopically altered mammary secretion. 7 subjects resulted healthy. The flocks were milked with an automated milking machine and monitored throughout the lactation period by means of the California Mastitis Test (CMT, Jorgensen Labs, Loveland, Colorado USA) and routine bacteriology due to persistent cases of mastitis<sup>9</sup>. The period of sampling reported in this work was at the end of the lactation period. Before samples were taken, the teats were carefully cleaned and the first three streams of foremilk were discarded. Ten milliliters were collected from each half udder and separated into two different sterile tubes containing 1 mL of glycerol (Merck, Darmstadt, Germany). After milking, teat swabs were taken. The milking machine swabs were put into

test tubes with Phosphate-buffered saline (PBS) and 10% glycerol. Four swabs were taken from the teat cups of the milking machine reserved for the infected animals. All samples were immediately placed in dry ice for transport to the laboratory, where they were stored at -20 °C.

### Bacteriological techniques

The clinical status of the flocks and hygienic conditions of the farms, was recorded by the “Istituto Zooprofilattico Sperimentale (IZS) della Sardegna”, an Italian public veterinary health institute that monitors the health status of dairy herds and also provides a free diagnostic service for farms. Bacteriological examination of biological samples (milk and swabs) was performed by plating according to standard methods. Each sample was cultured in sheep blood (5%) agar plate (Microbiol, Uta, Cagliari) and incubated in aerobic conditions, at 37 °C for 18-42 h. Pigmented colonies, showing a typical morphology and positive for the oxidase test (Bactident Oxidase, Merck, Germany) were considered as belonging to the *Pseudomonas* genus. Confirmation of identification was performed by means of a biochemical system API API20NE (BioMerieux, France) and ATB-GN system (BioMerieux France). In addition, further confirmation was performed by 16S rRNA sequencing using already described protocols. Briefly, DNA was extracted from putative Pa colonies by using the CTAB modified method<sup>10</sup>. PCR/sequencing oligos: OG33 (5' - GACTACCAGGGTATCTAATC - 3') and OG123 (5' - AGCAGCCGCGGTAATA - 3')<sup>8</sup> were used for PCR and for Sanger sequencing reaction<sup>11</sup>. Isolates were stored before the experiments in Tryptic Soy Broth (Microbiol, Uta, Cagliari), with glycerol at 15% at -80 °C. In addition to evaluating the susceptibility pattern to common antibiotics currently used in the human and veterinary field, we performed a susceptibility test for each strain by using the Vitek®-2 System (BioMerieux France) in accordance with the manufacturer's instructions<sup>12</sup>. We evaluated 14 different antibiotics for each strain: Piperacillin-tazobactam, Cefotaxime, Cefepime, Ertapenem, Imipenem, Meropenem, Amikacin, Gentamicin, Ciprofloxacin, Tigecycline, Fosfomycin, Nitrofurantoin, Colistin and Trimethoprim-sulfamethoxazole.

### *Pseudomonas aeruginosa* (Pa) strains isolated in this work

A total of 44 isolates of Pa were included in this study: 30 strains were isolated from milk; 13 were isolated from different points of the milking machine and 1 single strain (ID 23278) was isolated from farm well water (Table 1). The absence or presence of mucoid colonies was recorded for each colony, grown in Tryptic Soy Agar (TSA, Microbiol, Uta, Cagliari), to distinguish muc<sup>-</sup> and muc<sup>+</sup> phenotypes respectively. As controls, *P. aeruginosa* ATCC 15442 (American Type Culture Collection), recommended for disinfectant testing by official methods<sup>9</sup>, was used as the high biocide-resistant strain and *P. aeruginosa* ATCC 2783 was used as the susceptible strain.

### Susceptibility tests to disinfectants

Four different chemical compounds currently used in the Sardinian region for antimicrobial prophylaxis on farms were tested: (i) Chlorhexidine (CHX, Sigma, Country), diluted to obtain a 10% solution, equivalent to 100 mg/mL. (ii)

**Table 1** - Antimicrobial susceptibility tests (MIC) of 44 clinical isolates and 2 reference strains of *Pseudomonas aeruginosa*.

Sampling area	N° of strains	% muc <sup>+</sup> Pa	BZC	CHX	NaClO	H <sub>2</sub> O <sub>2</sub>
			µg/mL	µg/mL	µg/mL	ppm
Pabillonis	6	72% (Group I)	128	64	4096	250
San Gavino	3		128	64	2048	500
Bauladu	3		128	64	2048	500
Ortacesus	3		128	64	2048	250
Villaputzu	3		128	32	2048	500
Musei	3		128	16	4096	250
Villasor	3	50% (Group II)	64	64	4096	250
Serdiana	4		128	32	2048	250
Furtei	7	38% (Group III)	64	64	2048	250
Sanluri	5		64	32	1024	125
Codrongianos	3		64	16	2048	250
San Gavino*	1		64	8	2048	250
	1 ATCC 15442		128	128	4096	500
	1 ATCC 2783		64	8	2048	250

Legend: CHX = Chlorhexidine, BZC = Benzalkonium Chloride, NaClO = Sodium Hypochlorite, H<sub>2</sub>O<sub>2</sub> = Hydrogen Peroxide.  
\* = isolated from well water, strain 23278.

Benzalkonium chloride (BZC, SIGMA) 100 mg/mL. (iii) sodium hypochlorite (NaClO SIGMA), 1064 mg/L. (iv) Hydrogen Peroxide (H<sub>2</sub>O<sub>2</sub> Farve, Italy) 3%, (30000 ppm), the aliquots were stored at -20 °C. Following the commercial data sheets, we used the following concentration ranges: BZC and CHX from 128 to 0.25 µg/mL, NaClO from 8192 to 16 µg/mL and H<sub>2</sub>O<sub>2</sub> from 10000 to 1.95 ppm. All the consulted sheep-farmers reported using these antimicrobial products with this frequency order: CHX, NaClO, BZC and H<sub>2</sub>O<sub>2</sub>. CHX, NaClO were the most frequently used disinfectants in the area.

Before any experiment, 50 mL of the bacterial culture was inoculated onto a TSA plate. After 24 hrs. a single colony was inoculated into Muller Hinton Broth (Microbiol, Cagliari, Italy) and incubated at 37 °C for 15-20 hrs., until the growth middle log phase. A suspension of 10<sup>6</sup> CFU/mL bacterial cells was used as inoculum for all subsequent experiments. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were performed according to Clinical and Laboratory Standards Institute protocols (CLSI) using the micro-broth dilution method<sup>13</sup>. The MICs of the disinfectants were determined by serial 2-fold broth dilution in Mueller-Hinton broth of each product (Corning® 96 Well CellBIND® microplates, Merck, Germany). Each microdilution (100 µl/well) was inoculated with 100 µl of 10<sup>6</sup> CFU/mL of the overnight cultures. Then the microplates were incubated at 37 °C for 24 hrs. and the concentration of the disinfectant that totally inhibited the growth of the microorganisms was recorded as the MIC<sup>10</sup>. The highest MIC point was positioned at the values detected for the high resistant strain ATCC 15442.

The experiment was performed in triplicate and after 24 hours of growth at 37 °C, the turbidity at λ = 550 nm of each set of combinations was measured. For each compound, the MIC was the lowest concentration of an antimicrobial that inhibited visible growth<sup>10,11</sup>.

## Statistical analysis

Three distinct biological replicas were carried out for each analysis and quantitative data were expressed as mean ± SD. Pearson's chi-squared test was used to evaluate the significance between different analytical groups, i.e. susceptibility patterns of CHX, BZC and NaClO in comparison with increasing MICs of H<sub>2</sub>O<sub>2</sub> (P-values were set at p = 0.05).

## RESULTS AND DISCUSSION

On 11 ovine farms with recurrent and severe cases of acute mono lateral mastitis examined during the 2015-2017 lactation period, the veterinarians reported that the antibiotic treatment of these severe acute clinical cases had often been unsuccessful, even when antibiotic sensitivity testing had indicated that a particular drug should have been successful. The culture tests of milk, swabs and water demonstrated a consistence presence of *Pseudomonas aeruginosa*.

The phenotypic identification API API20NE (BioMerieux, France) was always confirmed by genotypic analysis (16S rRNA sequencing). The antibiotic susceptibility tests showed a multi-resistant profile for these clinical isolates. In fact, although all strains resulted sensitive to Aminoglycosides and Fluoroquinolones, all isolates resulted resistant to: Third-generation beta-lactams (Cefotaxime), Carbapenems, Tetracycline-class, Phosphonic acid derivative (Fosfomycin) and Trimethoprim-sulfamethoxazole. For other tested antibiotics the rate of resistance ranged from 10% to 80%. In this context a correct antimicrobial prophylaxis through disinfection could be crucial for mastitis forecasting in ovine breeding. For this reason, the present study has aimed to investigate disinfectant response in clinical ovine mastitis due to *P. aeruginosa* farm contamination. Moreover, we have also investigated the correlation between disinfectant MIC values and the presence of alginate coating *P. aeruginosa* colonies

(mucoid strains, muc<sup>+</sup>). In fact, as reported in the literature, the muc<sup>+</sup> strains resulted as being most resistant to antimicrobials in comparison with muc<sup>-</sup> phenotypes<sup>14</sup>. The experimental data summarized in Table 1 report the percentage of muc<sup>+</sup> strains on the farms and the respective MICs values of the 4 main disinfectants used on Sardinian sheep farms to treat milking machines, milk tanks and well water. In the light of the percentage of muc<sup>+</sup> strains, the farms were divided into three groups:

- **Group I.** 6 out of 11 farms (54.5%) showed a max MIC value for at least two studied disinfectants. 100% and 66.6% of the farms showed strains resistant to BZC and H<sub>2</sub>O<sub>2</sub> respectively.
- **Group II.** Highest MIC value for only one disinfectant (BZC), 2 breeding farms out of 11 (18.1%). The remaining disinfectants showed intermediate MIC values.
- **Group III.** Low or intermediate MIC values 27.4% of the studied farms.

The results shown in Table 1 suggest a correlation between the percentage of muc<sup>+</sup> strains and resistance to disinfectants, while no association has been observed between mucoid status and antibiotic resistance. In this case, all strains, except well water isolates, reported a MDR profile to antibiotics.

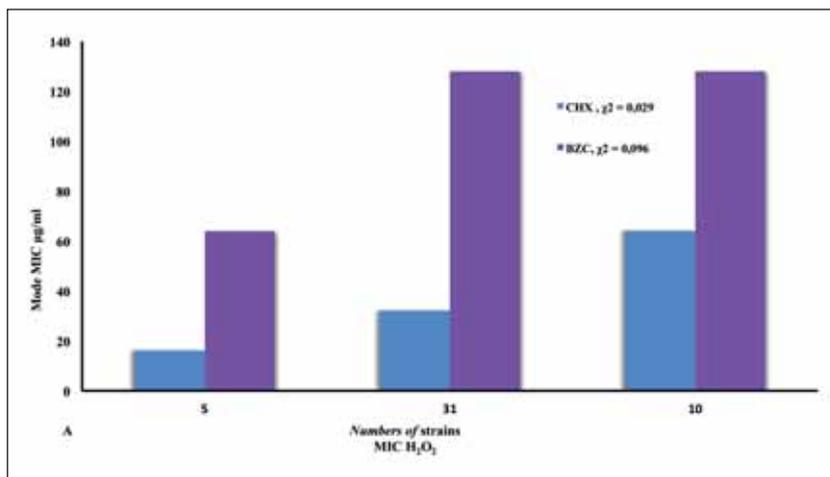


Figure 1A

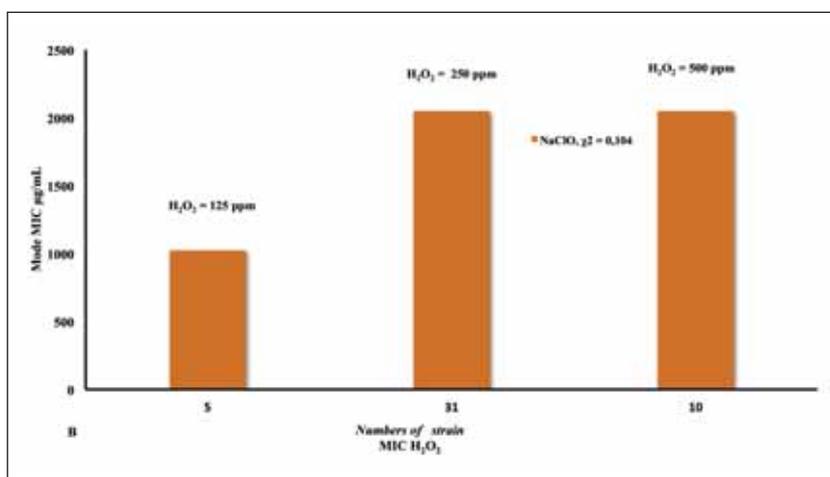


Figure 1B

**Figure 1** - Mode of MIC distribution for Chlorhexidine (CHX), Benzalkonium Chloride (BZC) and Sodium Hypochlorite (NaClO) in a series of *P. aeruginosa* strains ordered by H<sub>2</sub>O<sub>2</sub> increased MICs. Pearson's chi-squared index  $\chi^2$  were 0.029, 0.096, 0.104 for CHX, BZC and NaClO respectively.

Benzalkonium chloride (BZC) represented the compound to which Pa strains show more resistance on all breeding farms, with a high range of MIC values, from 128-to 64 µg/mL. This observation is in accordance with a previous experimental work, where BZC represented the most non-compliant disinfectant<sup>12</sup>. Previous experimental results found in the literature<sup>15</sup> suggest that in *P. aeruginosa* the decrease in the amount of adsorbed BZC is likely to be the result of increases in the contents of phospholipids (PL) and fatty and neutral lipids in the bacterial membrane and this bacterial adaptation to BZC could show resistance to other membrane-active agents but not to clinically relevant antibiotics. In this context, a non- statistical association, calculated with the Pearson  $\chi^2$  test was observed between BZC resistance and susceptibility to other studied disinfectants, except for Hydrogen Peroxide.

The results shown in Table 1 also suggest a non- homogeneous distribution of disinfectant susceptibility patterns among the various breeding places. In fact, the breeding farms were assigned to three different sensitivity groups by comparing the MIC values between high resistant ATCC 15442 and low resistant ATCC 2783 reference strains.

These results underline the emergence of antimicrobial resistance in *P. aeruginosa*, a problem that has been highlighted by the World Health Organization (WHO report 2017), with *P. aeruginosa* included in the "critical group", namely bacteria which pose a particular threat in hospitals, nursing homes and among patients whose care requires devices such as ventilators and blood catheters. In this manuscript we have focalized our attention on livestock animals, where erroneous antisepsis practices could be the reason for the appearance of drug resistant strains<sup>13</sup>. We have also speculated on the role of Hydrogen Peroxide as a disinfectant related to drug resistance in this bacterium. As showed in Figure 1, the mode MIC values are correlated with the MIC values for H<sub>2</sub>O<sub>2</sub> measured in all the analyzed strains, with high [H<sub>2</sub>O<sub>2</sub>] MIC values normally corresponding to high modes for all the compounds used in this study, for example a significant correlation was observed between H<sub>2</sub>O<sub>2</sub>- CHX, ( $\chi^2 = 0.029$ ).

This behavior could be explained by citing the results obtained in *P. aeruginosa* strains isolated from cystic fibrosis patients<sup>14</sup>. Mucoid phenotypes (muc<sup>+</sup>) among the strains infecting cystic fibrosis patients have indicated overproduction of the alginate, a linear polysaccharide situated in the bacterial capsule. These muc<sup>+</sup> strains are the cause of mortality in patients with cystic fibrosis. However, these authors demonstrated that after treating non- mucoid strains with low sub-inhibitory levels of H<sub>2</sub>O<sub>2</sub>, the formation of mucoid variants was observed. All muc<sup>+</sup> variants showed the same mutations in the *mucA* gene that encodes an an-

ti-sigma factor; this leads to the deregulation of an alternative sigma factor, required for expression of the alginate biosynthetic operon. The positive correlation between Hydrogen Peroxide MIC and susceptibility to other disinfectants could suggest the role of H<sub>2</sub>O<sub>2</sub> and the susceptibility of the other compounds used in this work. As reported in Table 1 and Figure 1, the MIC mode of the analyzed breeding farms was also related to the percentage of muc<sup>+</sup> isolates, 72% in high resistant strains. These results could be used to suggest the role of non-performant use of H<sub>2</sub>O<sub>2</sub> in breeding farm disinfection protocols with a possible use of sub-inhibitory concentrations of this disinfectant and the appearance of muc<sup>+</sup> strains in act the extensive alginate capsule in these bacteria is then responsible for host tissue adherence and low cell diffusion for various antimicrobials<sup>15</sup>.

## CONCLUSION

*P. aeruginosa* contamination/infection is not only related to the veterinary field but represents a global health problem. In fact, along with other bacterial species with zoonotic transmission, breeding procedures or uncorrected prophylaxis protocols could increase the virulence of these pathogens with an involvement in human health especially within nosocomial diseases. In this preliminary work we have investigated the antimicrobial profile of *P. aeruginosa* strains recruited from breeding farms with severe clinical cases of sheep mastitis. We observed a correlation between the Hydrogen Peroxide resistance profile, the presence of mucoid strains and the susceptibility patterns to 3 disinfectants, CHX, BZC and NaClO. In this study, NaClO appeared to be the most performant disinfectant. These results suggest a possible role of H<sub>2</sub>O<sub>2</sub> in the disinfection procedure in the Sardinian region as a “PA mucoid-converting compound” and a subsequent increase in the spread of high virulent strains in the environment. However, more studies are necessary to confirm this hypothesis such as *mucA* gene sequencing in these strains.

## FOOTNOTES/LINKS

WHO list of bacteria for which new antibiotics are urgently needed: <http://www.who.int/news-room/detail/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed>.

GenBank partial 16S rRNAs sequences from *P. aeruginosa* isolates were deposited with the following accession numbers: KU687333, KU647678, KU744948, KU877944, KU877945, KU877946, KU726585.

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