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MINIMAL INHIBITORY AND MUTANT PREVENTION CONCENTRATIONS OF  
ENROFLOXACIN FOR *PASTEURELLA MULTOCIDA* FROM RABBITS AFFECTED  
BY PASTEURELLOSIS

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## MINIMAL INHIBITORY AND MUTANT PREVENTION CONCENTRATIONS OF ENROFLOXACIN FOR *PASTEURELLA* *MULTOCIDA* FROM RABBITS AFFECTED BY PASTEURELLOSIS

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### ABSTRACT

*Pasteurella multocida* is the agent of one of the most significant diseases in rabbits and it is associated with a heterogeneous clinical picture. Drugs belonging to the fluoroquinolones class are useful to control pasteurellosis. Among them, enrofloxacin is one of the most used molecules in rabbit industry and it is the only one fluoroquinolone registered for this species in Italy.

Enrofloxacin adopted dosages are currently based on *Minimum Inhibitory Concentration* (MIC). Nevertheless, MIC is not effective against possible pathogen sub-populations with lower susceptibility that may be selectively amplified, leading to possible problems of antibiotic resistance. *Mutant Prevention Concentration* (MPC) could represent an approach to minimize the risk of resistance selection in pathogens. The aim of this work was to test the sensitivity to enrofloxacin of *P. multocida* strains isolated from rabbits affected by pasteurellosis to evaluate if MPC-based dosages can represent a valid option.

The study was performed on ten strains of *P. multocida* isolated from rabbits from two industrial farms of Puglia, South Italy. The sensitivity to enrofloxacin has been evaluated by MIC tests by microdilution method and MPC tests performed according to Marcusson et al. (2005) with minor modifications.

The results of MIC and MPC tests have revealed that MPC dosages are on average 8,4 times higher than MIC dosages. This data highlight that, although MPC-based dosages are useful to prevent the selection of potential mutant, they could be higher than MIC-based ones, leading to possible issues related to their application *in field*, for example the potential risk of possible toxicity for animals and residues in meat.

**Key words:** Rabbit, *Pasteurella multocida*, pasteurellosis, Minimum Inhibitory Concentration, Mutant Prevention Concentration

### INTRODUCTION

Pasteurellosis is one of the most significant disease of rabbits and causes considerable economic losses in large production units. Capsular types A, D and F of *Pasteurella multocida* have been reported in rabbits (Massacci et al., 2018). Several clinical signs, including respiratory problems, otitis, pyometra, orchitis, abscesses, torticollis, oculoconjunctivitis and septicaemia, are associated with pasteurellosis in rabbit (Palócz et al., 2014).

Enrofloxacin is one of the most used drugs in rabbit industry and it is useful to control diseases by pasteurellosis. Enrofloxacin is a fluoroquinolone approved in Europe for drinking water treatments in food producing rabbits. Although marbofloxacin, another fluoroquinolone, is very effective compared to enrofloxacin, danofloxacin, oxytetracycline or doxycycline against bacterial strains isolated from upper respiratory tract disease in rabbits (Rougier et al., 2006), it is not yet registered for the use in rabbit industry in Italy.

Considering the large number of rabbits usually reared in a single flock, the mass drug treatment by drinking water leads to expose sick rabbits but also healthy animals to drug, increasing the risk of development of antibiotic resistance in the bacteria.

The dosages of molecules for in field treatments are based on specific susceptibility parameters, i.e. the *Minimum Inhibitory Concentration* (MIC), which defines the drug efficacy against the susceptible pathogen population and leads to healing effects on rabbits. Nevertheless, MIC is not effective against possible sub-populations with lower susceptibility that may be selectively amplified (*Gehring and Riviere, 2013*).

A novel approach to minimize the risk of resistance selection is based on Mutant Prevention Concentration (MPC), which prevents the resistance selection in pathogens, including the potential mutants (*Zhao and Drlica, 2001*), that may be selectively amplified in a specific drug-concentration range, called Mutant Selection Window (MSW), between MIC and MPC values.

The aim of this study was to test the sensitivity to enrofloxacin of *Pasteurella multocida* isolated from rabbits affected by pasteurellosis and to evaluate if MPC-based dosages are a valid option for treatment.

## MATERIALS AND METHODS

### Origin of the strains

Totally, ten strains of *Pasteurella multocida* were tested. All the strains were isolated from different brood rabbits affected by pasteurellosis belonging to two different industrial rabbit farms of Puglia, South Italy. Four strains were from farm A and six from farm B. All strains were isolated on blood agar (5%) and identified by Multiplex-PCR according to *Townsend et al. (2001)*.

Each isolate was stored in Brian Heart Infusion Broth (BHIB) (OXOID) with glycerine (20%) at -20 °C until antimicrobial susceptibility tests were performed.

### Sensitivity test to enrofloxacin

MIC tests were performed by broth microdilution method according to CLSI guidelines (*CLSI, 2006*). The concentration of the *inoculum* ( $5 \times 10^5$  CFU/ml in well) was verified by spectrophotometer method at OD<sub>625</sub> (Spectrophotometer Beckman DU64a) and by plate counting after serial dilutions. Serial 2-fold dilution of antimicrobial agents were tested in concentrations ranging from 0.002 and 256 µg/ml. Drug, media and culture were added to each well of 96-well plates and statically incubated in aerobic condition at 37 °C for 24 h. Test on each isolate were performed at least three time in triplicate. Isolates were classified as susceptible (S: MIC  $\leq$  0.25 µg/ml) intermediate (I: MIC 0.5-1 µg/ml) or resistant (R: MIC  $\geq$  2 µg/ml) according to the ENRO breakpoints proposed by the CLSI (*CLSI, 2013*) for chickens and turkeys, because of the similarity in the breeding conditions and in the drug administration protocols between the two animal species.

MPC tests were performed as previously described by *Marcusson et al. (2005)* with minor modifications. Briefly, the tested strains were incubated in blood agar plates at 37°C overnight. All the plate content was transferred into 5 ml of Brian Heart Infusion Broth and incubated overnight at 37 °C. Then, 1 ml culture was transferred into 50 ml of BHIB and incubated at 37 °C for 4 h to obtain  $10^8$ - $10^9$  CFU/ml. The bacterial density was confirmed by plate counting. Aliquots of 10 ml of cultures were centrifuged at 1500xg for 15 min and the supernatant was discarded. The pellet (about  $10^{10}$  cfu) was re-suspended in the remaining liquid, spread on blood agar plates containing serial concentrations of the tested drug (1, 2, 4, 8, 16 and 32xMIC) and incubated at 37 °C for 72 h. MPC was defined as the lowest drug concentration at which no colonies were found. MPC was determined for each strain at least in three independent experiments. For each strain and drug, MPW and the mutant prevention index (MPI) were calculated as follows: MPW = MPC-MIC and MPI = MPC/MIC.

## RESULTS AND DISCUSSION

All tested strains were *in vitro* sensitive to enrofloxacin, based on the susceptibility break points ( $S \leq 0.25 \mu\text{g/ml}$ ) (CLSI, 2013), proposed for cattle, swine and poultry. In fact, specific susceptibility break points for rabbit are not available, currently. According to the obtained results, resistance was rarely found in *Pasteurella multocida* strains isolated from diseased food producing animals and pet (Bourély *et al.*, 2019). In the tested strains, the MIC values ranged from 0.004 to 0.125  $\mu\text{g/ml}$  (Table 1).

**Table 1:** Sensitivity values to enrofloxacin for *P. Multocida* strains from rabbits

Farm	Strain	MIC ( $\mu\text{g/ml}$ )	MPC ( $\mu\text{g/ml}$ )	MPI (MPC/MIC)	MSW (MPC-MIC) ( $\mu\text{g/ml}$ )
A	1 (344)	0.125	0.5	4.00	0.375
	2 (338)	0.03	0.125	4.17	0.095
	3 (343)	0.015	0.125	8.33	0.11
	4 (337)	0.125	0.5	4.00	0.375
B	5 (346)	0.03	0.5	16.67	0.47
	6 (349)	0.004	0.03	7.50	0.026
	7 (348)	0.004	0.06	15.00	0.056
	8 (353)	0.03	0.25	8.33	0.22
	9 (347)	0.03	0.25	8.33	0.22
	10 (345)	0.03	0.25	8.33	0.22
Mean		0.04	0.259	8.466	0.2167

No relevant differences were observed among the strains according to the farm origin.

Particularly, MIC values resulted 0.004, 0.03, 0.015 and 0.125  $\mu\text{g/ml}$ , depending on the different strains, with a mean value corresponding to 0.04  $\mu\text{g/ml}$ . Studies carried out on *P. multocida* isolated from swine (Blondeau and Fitch, 2019) highlighted similar data (MIC values  $\leq 0.016$ ) to those on average found in the tested strains from rabbits.

Notwithstanding the *in vitro* sensitivity of *P. multocida* to enrofloxacin, *in vivo* therapies are not generally able to eradicate the infection (Mähler *et al.*, 1995). Besides, the severity of lesions due to the bacteria and the heterogeneity of clinical pictures, that sometimes cause difficulty for the drug to reach some target tissue as often occurs in presence of abscesses for example, can lead to inefficacy of the therapy.

The mean MPC value resulted 0.259  $\mu\text{g/ml}$ , with a range of 0.03-0.5  $\mu\text{g/ml}$ . MSW ranged from 0.026 to 0.375  $\mu\text{g/ml}$ . Interestingly, the MIP value reached more than 15 times higher than MIC values for two strains, while it resulted of 8.4 times higher than MIC values on average, considering all the tested strains.

These results seem to indicate that the MPC-based dosages useful to prevent the selection of potential mutant should be higher than MIC-based dosages. The latter are not enough to eliminate the bacterium and potentially can lead to the selection of potential mutant. Therefore, dosages of the drug used in field which are based on MIC values may be useful to obtain an improvement of clinical form but often lead to the persistence of the infection increasing the potential risk of selection of mutant more resistant. Nevertheless, MPC-based dosages are not suitable for field application, to our opinion. Firstly, they are in contrast with the appropriate antibiotics use guidelines, that suggest reducing the use of drugs in animal flocks for food production. Moreover, *in field* adopted dosages are currently based on MIC and there are no available data about enrofloxacin potential toxicity and residues related to MPC-based dosages. For this reason, the adoption of such a high amount of drug could lead to several issues, for example possible risks of toxicity for the animals, as well as of problems of antibiotic traces in meat with consequent possible increases of suspension period before slaughter.

Therefore, alternative strategies should be considered in order to control the disease in farms, including enhanced management and hygienic conditions, the more frequent use of vaccines against *P. multocida* and other common infectious agents in rabbits, the selection of genetic lines more resistant to the infection.

## CONCLUSIONS

The results obtained from this study show that enrofloxacin MPC-based values could be more than 8 times higher than MIC-based values. Therefore, the dosages based on MIC currently adopted *in field* lead to improvements of clinical signs in farm but may lead to the selection of strains more resistant to drug. On the upside, the adoption of MPC-based dosages in field is in contrast with the appropriate use of antibiotics guidelines and, to our opinion, could lead to possible risks of toxicity on animals, possible increase of residues in meat and a possible decrease of the palatability of medicated water. Therefore, this study suggests the necessity to adopt alternative strategies, such as the selection of genetic lines more resistant to the infection, a more frequent use of vaccines against *P. multocida*, to control the pasteurellosis in rabbit farms.

## ACKNOWLEDGEMENTS

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# Minimal Inhibitory and Mutant Prevention Concentrations of Enrofloxacin for *Pasteurella multocida* from rabbits affected by pasteurellosis

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Respiratory disorders



Encephalitis

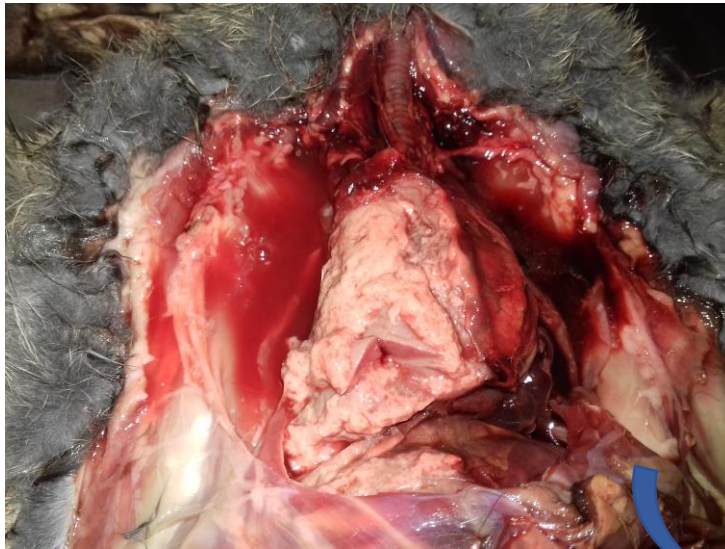


Otitis



Serogroups  
responsible  
for:  
A, D, F

Pneumonia



Pyometra

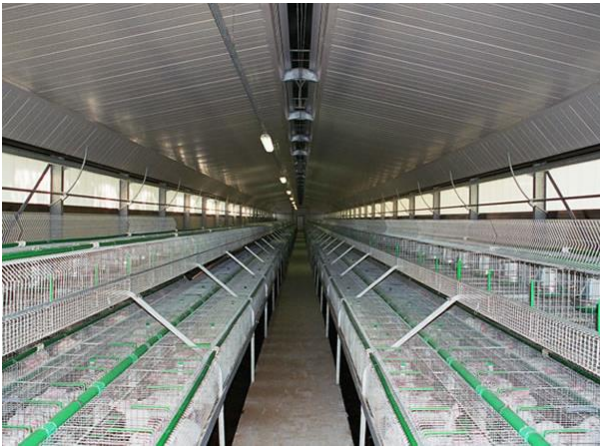
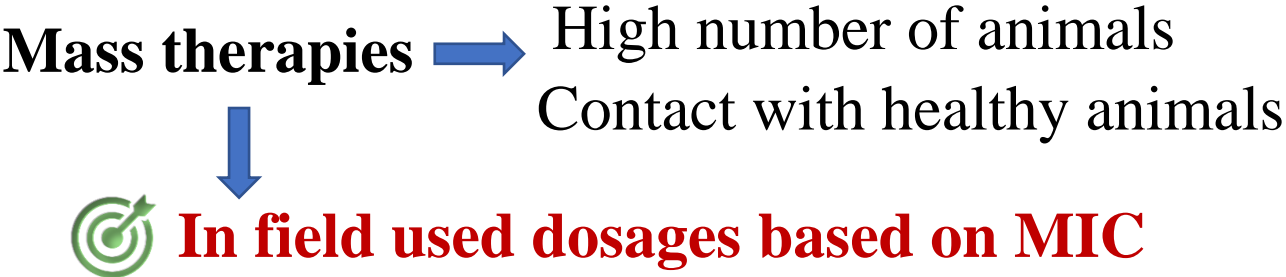


Mortality

Disease influenced by predisposing factors: density, dust, ammonia, hygienic conditions

# ENROFLOXACIN

Approved in Europe for drinking water treatments



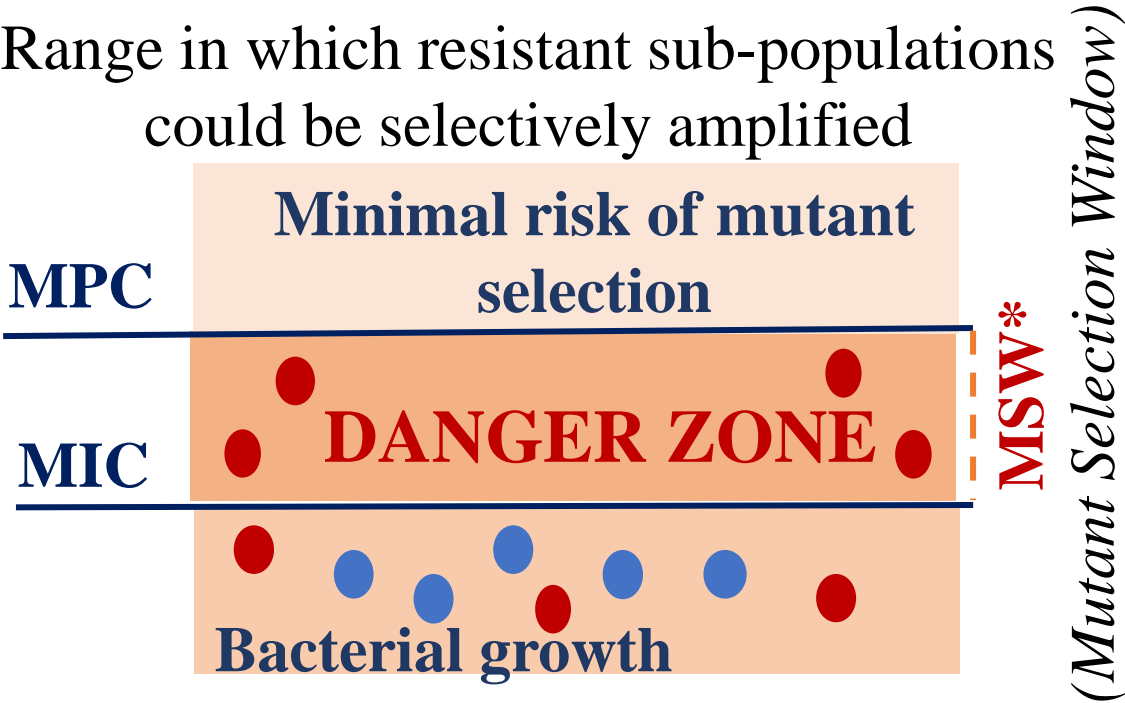
MIC (*Minimal Inhibitory Concentration*)



Clinical healing      Selection prevention



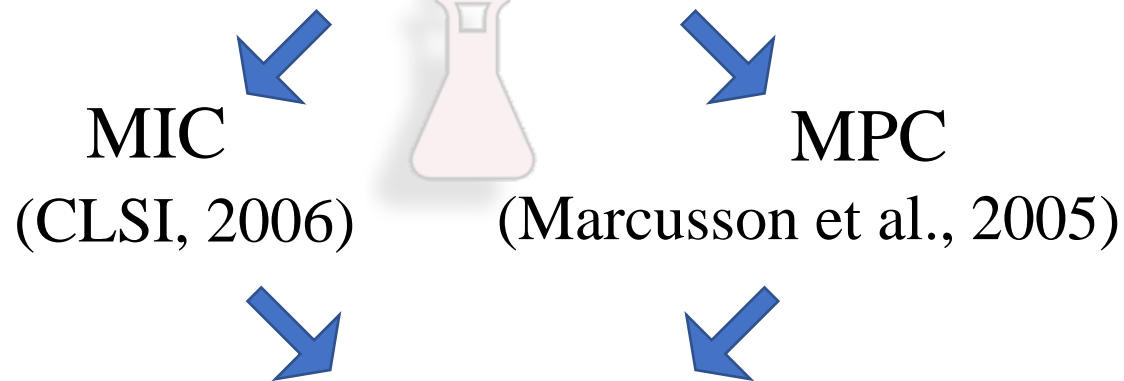
MPC (*Mutant Prevention Concentration*)





## Aim of the study

- Test the sensitivity to enrofloxacin of strains from rabbits dead by pasteurellosis



MSW (Mutant Selection Window)

**MPC - MIC**

MPI (Mutant Prevention Index)

**MPC / MIC**

## Materials and methods

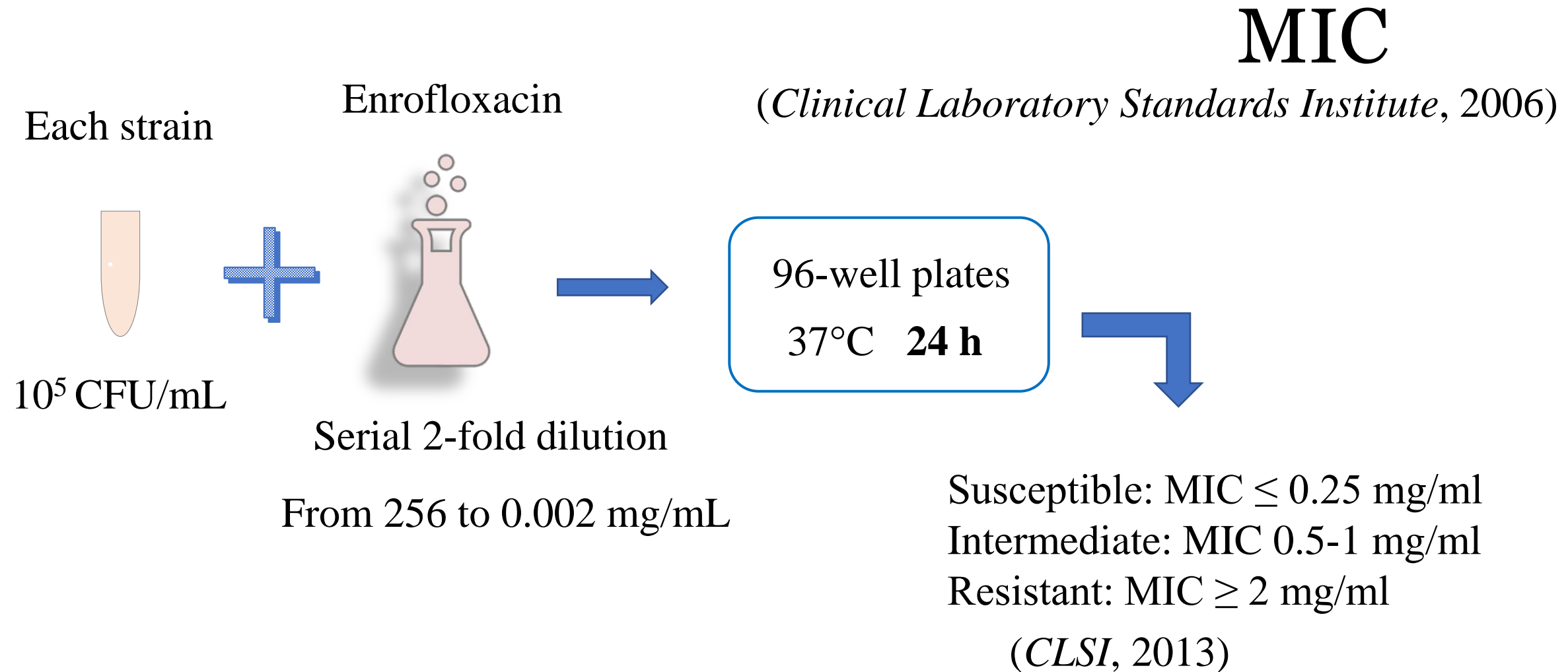
10 *Pasteurella multocida* strains (ser. A)

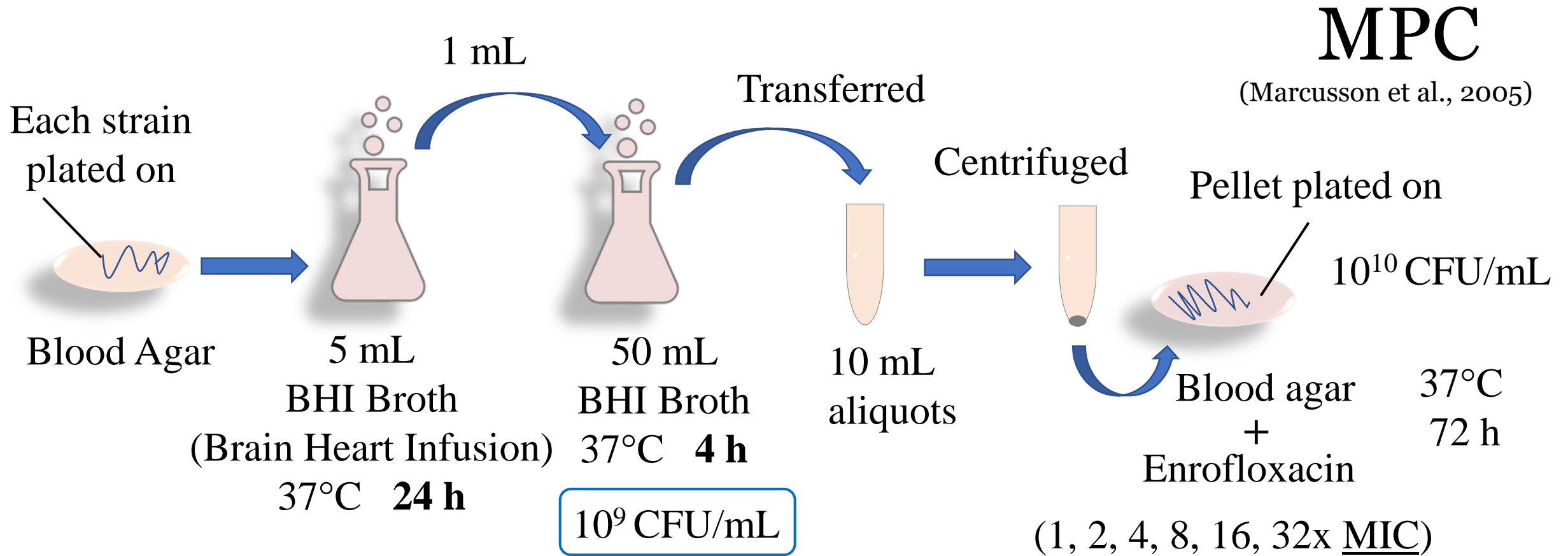


Farm A (4 strains)

Farm B (6 strains)







**MPC** = lowest drug concentration at which no colonies are found

## Results

### Sensitivity values to enrofloxacin

		Strain	MIC ( $\mu\text{g/ml}$ )	MPC ( $\mu\text{g/ml}$ )
Farm A	{	1	0.125	0.5
		2	0.03	0.125
		3	0.015	0.125
		4	0.125	0.5
Farm B	{	5	0.03	0.5
		6	0.004	0.03
		7	0.004	0.06
		8	0.03	0.25
		9	0.03	0.25
		10	0.03	0.25
		Mean	0.04	0.259

Variable values depending on strains

- **MIC ranging from 0.004 to 0.125**  
From 0.015 to 0.125 (farm A)  
From 0.004 to 0.03 (farm B)
- **MPC ranging from 0.03 to 0.5**  
From 0.125 to 0.5 (farm A)  
From 0.03 to 0.5 (farm B)

## Results

### Sensitivity values to enrofloxacin

	Strain	MIC ( $\mu\text{g/ml}$ )	MPC ( $\mu\text{g/ml}$ )
Farm A	1	0.125	0.5
	2	0.03	0.125
	3	0.015	0.125
	4	0.125	0.5
Farm B	5	0.03	0.5
	6	0.004	0.03
	7	0.004	0.06
	8	0.03	0.25
	9	0.03	0.25
	10	0.03	0.25
	Mean	0.04	0.259

### Variable values depending on strains

MPC / MIC	MPI	MSW ( $\mu\text{g/ml}$ )	MPC - MIC
<div>At least 4 times</div> <div>More than 16 times for one strain!!</div>	4.00	0.375	
	4.17	0.095	
	8.33	0.11	
	4.00	0.375	
	16.67	0.47	
	7.50	0.026	
	15.00	0.056	
	8.33	0.22	
	8.33	0.22	
	8.33	0.22	
Mean	8.466	0.216	





Is MPC-based dosage a valid option?

**Too high dosages!**



Possible risk  
of toxicity



Possible increase  
of residues



Possible effects  
on palatability

## Alternative strategies



Improved  
hygienic  
conditions



Increasead use of  
vaccines for  
bacterial diseases



More resistant  
genetic lines for  
the disease



*Real Pasteurella multocida!!*

*Thank you for your attention!*



*“Fake” Pasteurella multocida!!*

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