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# Microbiological Method using *Bacillus megaterium* with Fusidic Acid for Detection of Macrolides in Milk

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**Abstract**

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The microbiological method to attain a sensitive detection of macrolides using *Bacillus megaterium* in agar medium with fusidic acid was designed. To this aim, Mueller-Hinton medium fortified with glucose at pH 8.0, a combination of redox indicators (brilliant black and toluidine blue) and different concentrations of fusidic acid were tested. The addition of fusidic acid in the culture medium improves the sensitivity of this bacteria test and decreases the detection limits of bioassay. The addition of 200 µg/l of fusidic acid detects 35 µg/l of erythromycin, 58 µg/l of tylosin, and 57 µg/l of tilmicosin in milk. This microbiological bioassay could be used as an alternative method of commercial screening test for detecting macrolides in milk, in order to maintain food safety.

**Keywords**: microbiological test; antibiotic; erytromycin; tylosin; tilmicosin

Macrolides (MC) are bacteriostatic compounds effective against a wide variety of Gram-positive bacteria (Shiomi & Omura 2002), but have limited activity against Gram-negative bacteria (Edder *et al*. 2002). These molecules bind to the 23S rRNA bacterial ribosome domain in a reversible way, causing the inhibition of protein synthesis (Douthwaite & Champney 2001; Zhanel *et al*. 2001).

These antibiotics (ATBs) are used in veterinary medicine, mainly for the treatment of diseases of the upper respiratory tract, bronchial pneumonia, enteritis, metritis, urinary infections, and arthritis, among others. In particular, erythromycin is provided for treating clinical and subclinical mastitis in lactating cows (Wang & Leung 2007). Inappropriate use of MC or insufficient withdrawal time increases the probability of finding their residues in animal products, including milk (Edder *et al.* 2002). Antibiotic residues can cause toxic effects in consumers, for example allergic reactions or induction of antimicrobial resistance, e.g. *Streptococcus pyogenes* (Dixon and Lipinski 1974), *Campylobacter jejuni* (Burridge *et al.* 1986), *Mycoplasma pneumoniae* (Stopler & Branski 1986), *Lactobacillus* spp. (Rinckel & Savage 1990), *Staphylococcus* spp. (Moats & Medina 1996), and members of *Enterobacteriaceae* (Mulazimoglu *et al.* 2005).

For the purpose of maintaining the health of consumers, control authorities established Maximum Residue Limits (MRLs) of 40 µg/l for erythromycin and 50 µg/l for tylosin or tilmicosin (Council Directive 2009; Codex Alimentarius 2010).

Many screening tests with *G. stearothermophilus* have been developed for precise detection of antibiotic residues in milk (Toldra & Reig 2006; IDF 2010). These tests are not sensitive enough to detect antibiotics such as quinolones (Montero *et al.* 2005), spiramycin, lincomycin (Linage *et al.* 2007), erythromycin, and streptomycin (Althaus *et al.* 2003; Molina *et al.* 2003).

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Thus, for the specific control of MC residues, some authors propose the use of *Kocuria rhizophila* as the bacteria test in Petri dishes (Nouws *et al.* 1999; Pikkemaat *et al.* 2008, 2009, 2011; Althaus *et al.* 2009). However, the methods that use vegetative cells in Petri dishes are difficult to implement as a routine technique in a residue control laboratory because they require the prior preparation of a method with cells in exponential phase. Also, this microbiological test in Petri dish shows long incubation times (16–24 h) and low conservation period at refrigerator temperature.

Therefore, the purpose of this study was to develop and optimise a microtiter plate bioassay using *Bacillus megaterium* spores with fusidic acid (FA) to detect MC in milk in a sufficiently short time period. The method could process easily a large number of milk samples due to its design in microtiter plate with 96 wells.

## MATERIAL AND METHODS

***Bioassay elaboration***. Mueller-Hinton Agar culture medium (38 g/l, Ref. 10272; Biokar Diagnostics, Allonne, France) was prepared at pH 8.00 ± 0.1 fortified with 10 g/l of glucose (Ref. G8270; Sigma-Aldrich®, St. Louis, USA), 200 mg/l of brilliant black (Ref. 211842; Sigma-Aldrich®), and 10 mg/l of toluidine blue (Ref. 89640; Sigma-Aldrich®). Inoculations with different *B. megaterium* (ATCC 9885; American Type Culture Collection, Manassas, USA) spore concentrations and fusidic acid (Ref. F0756; Sigma-Aldrich®) were performed as detailed for each experiment. The concentration of the stock spore suspension ($5.6 \times 10^{10}$ spores/ml, Log S = 10.75) was estimated with Petrifilm™ plates (3M, St. Paul, USA). A volume of 100 µl of culture medium was added to each microplate well using an electronic dispenser (Eppendorf Research® Pro, Hamburg, Germany). Bioassay plates were sealed with aluminised polypropylene bands and refrigerated at 4°C until use.

***Analysis of dose-response curves***. Sixteen replicates of twelve concentrations (detailed in each experiment) were analysed for each antibiotic, so as to obtain at least two negative results at the lowest concentrations and two positive results at the highest levels.

For this, a volume of 50 µl of a solution containing the milk and the corresponding antibiotic concentration was added to each microplate well and left at 25°C for 1 h to diffuse antimicrobial substances in the agar medium of the bioassay. Later, the microplates were washed 3 times with distilled water to remove the remaining milk and placed in a water floating bath at 45 ± 1°C (Dalvo, Santa Fe, Argentina) until a change in the colour of the negative control samples (from black to yellow). During the growth of the bacteria test, reduction processes are developed and consequently the redox indicator (brilliant black and toluidine blue) changes from black (original) to yellow (growth) colour.

Then, photometric readings were performed with ELISA microplate reader (Biotek ELx800™; Biotek Instrument Inc., Winooski, USA) at 550 nm. The results were transformed in terms of relative absorbances according to the following transformation:

$$A = (A_x - A_0)/(A_{100} - A_0) \quad (1)$$

where: A – relative absorbance; $A_x$ – absorbance of the milk sample with an x antibiotic concentration; $A_0$ – absorbance of antibiotic-free milk (negative control); $A_{100}$ – absorbance of the milk sample that yielded 100% positive results

These relative absorbance values were analysed using the logistic procedure of the statistical package StatGraphics Plus Centurión®, Version 16 (StatGraphics, 2008). Then, detection limits were calculated as the concentration of antibiotic that produces 45% of relative absorbance (Nagel *et al.* 2011).

***Effect of spore concentration on response time and detection limits of the bioassay***. Culture medium was divided into five aliquots to evaluate the effect of different percentages of stock spore suspension (logarithm of concentration in each aliquot): 0.008% (Log S = 6.6), 0.04% (Log S = 7.3), 0.2% (Log S = 8.0), 1% (Log S = 8.7), and 2% (Log S = 9.1). Tylosin concentrations used were 0, 50, 75, 100, 125, 150, 175, 200, 250, 300, 400, and 500 µg/l. The response time was determined when negative control samples changed their colour (from blue to yellow). These results were adjusted using a negative exponential regression model.

***Effect of fusidic acid concentration on bioassay detection limits***. The culture medium was inoculated with $2.8 \times 10^8$ spores/ml of *B. megaterium* and was divided into 4 aliquots to analyse the effect of fusidic acid (FA) 0, 100, 150, 200 µg/l. For each FA level, 16 replicates of 12 concentrations of erythromycin (Sigma E-6376, St. Louis: 0, 10, 15, 20, 30, 40, 50, 60, 80, 100, 150, and 200 µg/l), tylosin (Sigma T-6134: 0, 10, 20, 30, 40, 50, 60, 80, 100, 120, 150, 200 µg/l) and tilmicosin (Vetranal 33864™; Sigma-Aldrich, St. Louis, USA – 0, 10, 20, 30, 40, 50, 60, 80, 100, 120, 150, 200 µg/l) were tested. Bioassays were incubated

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at 45 ± 1°C for 5 hours. The logistic regression model used was as follows:

$$L_{ijk} = \text{Logit}[A_{ijk}] = \beta_0 + \beta_1[MC]_j + \beta_2[FA]_j + \beta_{12}([MC]^*[FA])_{ij} + \varepsilon_{ijk} \quad (2)$$

where: $L_{ijk}$ – linear logistic model; $[A_{ijk}]$ – relative absorbance (Eq. 1); $[MC]_i$ – macrolide concentration ($i$ = 1, 2, ..., 12 levels); $[FA]_j$ – FA concentration ($j$ = 50, 100, 150, 200 µg/l); $([MC]^*[FA])_{ij}$ – interaction between MC and FA concentrations; $\beta_0$, $\beta_1$, $\beta_2$, $\beta_{12}$ – coefficients estimated for the model; $\varepsilon_{ijk}$ – residual error

***Bioassay specificity***. 192 individual milk samples from Holstein cows that had not received any antimicrobial treatment during lactation were analysed by triplicate using bioassays containing 0 and 200 µg/l of FA. Responses were interpreted visually (negative or positive) by three qualified persons. For statistical analyses, the visual results which have at least two similar performances were considered. The specificity was calculated according to the following expression:

$$\text{Specificity} = (\text{negative samples/total samples}) \times 100 \quad (3)$$

***Bioassay cross-specificity***. Bioassays were developed with $2.8 \times 10^9$ spores/ml of *B. megaterium* and 200 µg/l of FA. Detection limits of eight β-lactams (amoxicillin, ampicillin, cloxacillin, oxacillin, benzylpenicillin, cefoperazone, ceftiofur, and cephalexin), three aminoglycosides (kanamycin, neomycin, and streptomycin), three tetracyclines (chlortetracycline, oxytetracycline, and tetracycline), four sulfonamides (sulfadiazine, sulfadimethoxine, sulfamethoxazole, and sulfathiazole), and three quinolones (ciprofloxacin, enrofloxacin, and marbofloxacin) were evaluated. For each antimicrobial agent, sixteen replicates of all concentrations (12) were tested. Results were analysed using a logistic regression model:

$$L_{ij} = \text{Logit}[A_{ij}] = \beta_0 + \beta_1[ATB]_j + \varepsilon_{ij} \quad (4)$$

where: $L_{ij}$ – logistic linear model; $[A_{ij}]$ – relative absorbance; $[ATB]_i$ – antibiotic concentration ($i$ – 1, 2, .... 12 levels); $\beta_0$, $\beta_1$ – estimated coefficients; $\varepsilon_{ij}$ – residual error

## RESULTS AND DISCUSSION

***Effect of spore concentration on the bioassay response time***. Increases in spore concentration (Log S = 6.6, 7.3, 8.0, 8.7, and 9.0) caused decreases in the bioassay response time ($t$ = 8.5, 6.0, 5.0, 4.5, and 4.4 h, respectively), which was also observed by Nagel *et al.* (2011) in the optimisation of a bioassay using *Bacillus cereus* for tetracycline detection in milk. The equation that relates the time ($t$) and the logarithmic transformation of the spore concentration (Log S) was as follows: $t$(h) = $\exp^{(-0.3551 + 16.108/\text{Log S})}$. The quadratic correlation coefficient was high ($R^2$ = 98.1%) indicating an adequate model fit. Spore concentrations greater than Log S = 8.7 did not cause a significant decrease in the response time. Therefore, this spore concentration (Log S = 8.7) was used in successive experiments.

***Effect of fusidic acid on the bioassay detection limits***. The macrolide concentration (MC) was significant for all three molecules tested ($P < 0.0001$). The interaction between macrolides and fusidic acid concentrations [MC]*[FA] was significant for erythromycin ($\chi^2$ =11.341, $P$ = 0.0008) and tylosin ($\chi^2$ = 20.937, $P$ = 0.0001) indicating a synergistic effect for these two MC. However, [FA] showed a significant effect with tilmicosin ($\chi^2$ = 22.096, $P$ = 0.0001), but the [MC]*[FA] interaction was not significant for this antibiotic ($\chi^2$ = 0.105, $P$ = 0.774).

A synergic effect between erythromycin and FA was observed by Garrett and Richards (1974) with different pathogenic microorganisms, although they did not evaluate this possible interaction with other MC, such as tylosin and tilmicosin.

Table 1 summarises the logistic regression equations with the significant effects in the model (Eq. 2). Concordance percentages were adequate (erythromycin = 93.3%; tilmicosin = 95.3%; tylosin = 93.3%). The $\beta_1$ coefficient indicates the increase in relative absorbance due to crescent MC concentrations in milk. This coefficient indicates that *B. megaterium*

Table 1. Logistic regression equations representing the effect of macrolide concentration and fusidic acid levels on the bioassay relative absorbance

| Macrolides | $L = \beta_0 + \beta_1[MC] + \beta_2[AF] + \beta_{1-2}[MC]^*[AF]$ | C% |
|---|---|---|
| Erythromycin | $L = -2.2094 + 0.0359[MC] + 0.0001[MC]^*[AF]$ | 93.3 |
| Tilmicosin | $L = -4.7282 + 0.0484[MC] + 0.0088[AF]$ | 95.3 |
| Tylosin | $L = -3.3749 + 0.0335[MC] + 0.0001[MC]^*[AF]$ | 93.3 |

L – Logistic model; MC – macrolides; FA – fusidic acid; C% – concordance percentage

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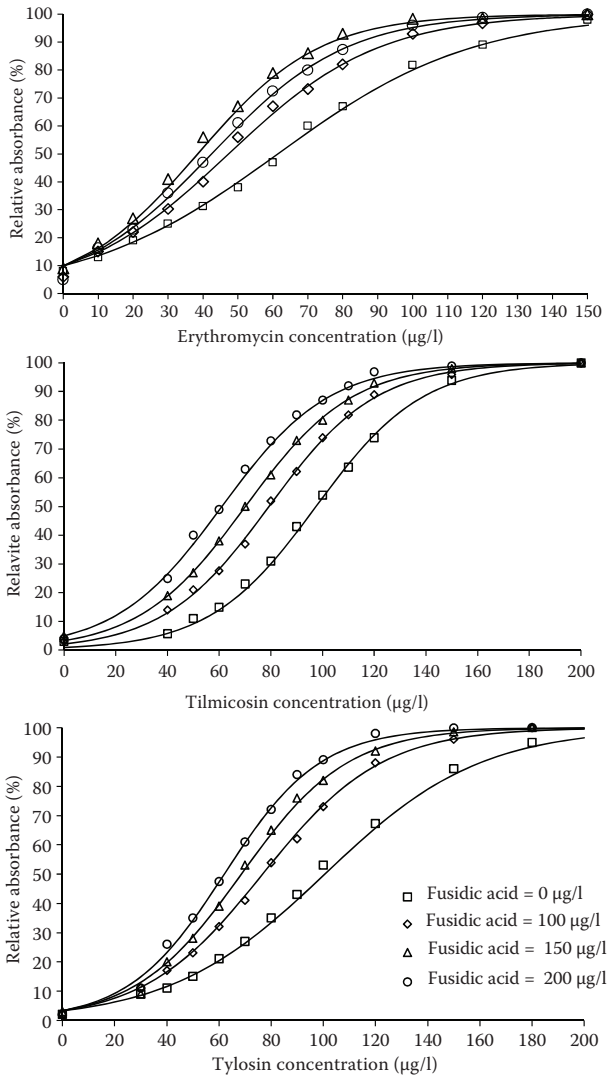


Figure 1. Effect of fusidic acid on the dose-response curves of macrolides in milk

has good sensitivity to detect this three MC in milk, since $\beta_1$ values were high (erythromycin = 0.0359; tilmicosin = 0.0485, and tylosin = 0.0336). The $\beta_2$ coefficients show the effect of FA for tilmicosin ($\beta_2$ = 0.0088), while $\beta_{1-2}$ coefficients describe the effect of the [MC]*[AF] interaction for erythromycin ($\beta_{1-2}$ = 0.0001) and tylosin ($\beta_{1-2}$ = 0.0001). The significant effects ([MC], [FA], and/or [MC]*[FA]) on the relative absorbance of the bioassay are shown in Figure 1. It is observed that crescent MC concentrations in milk resulted in increased relative absorbance. The addition of FA causes displacements of the logistic curve to detect lower concentrations, indicating an improvement in the sensitivity of the bioassay. The MC detection limits obtained for different levels of FA in bioassays with their respective MRLs are shown in Table 2. Adding FA to the culture medium (from 0 to 200 µg/l) causes a decrease in the detection limit of erythromycin (46–35 µg/l), tylosin (95–58 µg/l), and tilmicosin (94–57 µg/l) at levels similar to their MRLs.

The use of *B. megaterium* in Petri dishes (45°C, 18 h) allowed the detection of 30 µg/l of erythromycin in kidney fluid (Dey *et al.* 2005), which is similar to 35 µg/l detected at 6 h with the bioassay optimised in this work (Table 2). The microbiological methods in Petri dishes that used *Kocuria rhizophila* as a bacteria test specific for MC (30°C, 24 h) cannot detect residues of the main MC used frequently in the therapeutic of dairy cattle. Thus, Nouws *et al.* (1999) detected 30 µg/l of erythromycin and 10 µg/l of tilmicosin in cow milk, but failed to detect tylosin (500 µg/l) at levels close to their MRL (50 µg/l). Similarly, Tsai and Kondo (2001) detected low levels of erythromycin (50 µg/l) and achieved high minimum detectable concentrations for tylosin (390 µg/l) residues in milk when using *Kocuria rhizophila*. In the same way, Gaudin *et al.* (2004) detected residues of erythromycin (30 µg/l) and tilmicosin (50 µg/l) but they did not obtain any adequate minimum inhibitory concentrations for tylosin (200 µg/l) in milk. In sheep's milk, Althaus *et al.* (2009) determined good minimum inhibitory concentration of erythromycin (80 µg/l) and high concentration of tylosin (1000 µg/l) in Petri dish that uses the same bacteria test.

In meat matrices, Pikkemaat *et al.* (2008) calculated the detection capacity (CCβ) of 150 µg/l for erythromycin, 400 µg/l for tylosin, and 300 µg/l for tilmicosin when they used renal pelvis fluid with the specific MC plate (*K. rhizophila*) that integrates the NAT (Nouws Antibiotic Test) post-screening in

Table 2. Effect of fusidic acid on macrolide detection limits in milk

| Macrolides | Concentration FA (µg/l) | | | | MRLs (µg/l) |
|---|---|---|---|---|---|
| | 0 | 100 | 150 | 200 | |
| Erythromycin | 46 | 44 | 39 | 35 | 40 |
| Tilmicosin | 95 | 73 | 65 | 58 | 50 |
| Tylosin | 94 | 75 | 66 | 57 | 50 |

FA – fusidic acid; MRLs – Maximum Residue Limits

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Table 3. Coefficients of the logistic regression model for dose-response curves of the bioassay

| Antibiotic | Logit [P] = $\beta_0$+ $\beta_1$ [ATB] | C% | DL | MRLs |
|---|---|---|---|---|
| **Beta-lactam** | | | | |
| Amoxicillin | L [P] = –0.7484 + 0.0065 [ATB] | 85.8 | 85 | 4 |
| Ampicillin | L [P] = –1.5402 + 0.0020 [ATB] | 95.9 | 76 | 4 |
| Cloxacillin | L [P] = –1.3340 + 0.0049 [ATB] | 83.1 | 228 | 30 |
| Oxacillin | L [P] = –1.3329 + 0.0049 [ATB] | 82.1 | 250 | 30 |
| Penicillin | L [P] = –1.7180 + 0.0053 [ATB] | 82.4 | 287 | 4 |
| Cephalexin | L [P] = –2.5631 + 0.0029 [ATB] | 94.5 | 812 | 100 |
| Cefoperazone | L [P] = –2.2783 + 0.0009 [ATB] | 92.9 | 2158 | 50 |
| Ceftiofur® | L [P] = –2.3120 + 0.0035 [ATB] | 74·5 | 598 | 100 |
| **Aminoglycosides** | | | | |
| Kanamycin | L [P] = –1.0657 + 0.0012 [ATB] | 86.2 | 670 | 150 |
| Neomycin | L [P] = –0.5384 + 0·0024 [ATB] | 72.5 | 550 | 1500 |
| Streptomycin | L [P] = –0.8063 + 0.0010 [ATB] | 77.5 | 600 | 200 |
| **Tetracyclines** | | | | |
| Chlortetracycline | L [P] = –0.7870 + 0.0032 [ATB] | 76.8 | 185 | 100 |
| Oxytetracycline | L [P] = –1.4974 + 0.0078 [ATB] | 89.6 | 170 | 100 |
| Tetracycline | L [P] = –1.6921 + 0.0070 [ATB] | 82.8 | 213 | 100 |
| **Sulfonamides** | | | | |
| Sulfadiazine | L [P] = –1.4294 + 0.0002 [ATB] | 87.7 | 5800 | 100 |
| Sulfadimethoxine | L [P] = –2.4032 + 0.0005 [ATB] | 94.3 | 4380 | 100 |
| Sulfamethoxazole | L [P] = –1.9999 + 0.0008 [ATB] | 88.5 | 2100 | 100 |
| Sulfathiazole | L [P] = –2.1719 + 0.0005 [ATB] | 83.8 | 3900 | 100 |
| **Quinolones** | | | | |
| Ciprofloxacin | L [P] = –1.6426 + 0.0037 [ATB] | 92.3 | 390 | 50 |
| Enrofloxacin | L [P] = –2.5559 + 0.0027 [ATB] | 96.2 | 848 | 100 |
| Marbofloxacin | L [P] = –2.8595 + 0.0034 [ATB] | 94.4 | 767 | 75 |

L [P] – Logistic model; ATB – antibiotic; C% – concordance percentage; DL – detection limit; MRLs – Maximum Residue Limits (µg/l)

16–18 hours. In porcine and bovine kidney samples, Pikkemaat *et al*. (2009) estimated CCβ of 30 µg/l for erythromycin, 50 µg/l for tylosin, and 60 µg/l for tilmicosin for the NAT screening method (16–18 h). Whereas Gaudin *et al*. (2010) detected 200 µg/l of erythromycin and 100 µg/l of tylosin when they analysed raw muscle using the STAR (Screening Test for Antibiotic Residues) protocol.

Therefore, it would be interesting to evaluate the performance of the bioassay in other matrices (goat milk, sheep milk, beef, chicken, etc.), since it presents low detection limits for macrolide used in the livestock treatment.

***Bioassay specificity***. The analysis of 192 milk samples from untreated animals indicated 2 (98.9%) and 6 (96.8%) positive cases for bioassays developed with 0 and 200 µg FA/l, respectively. For Delvotest® commercial methods, Sischo and Burns (1993), and Charm and Zomer (1995) obtained the specificity of 98 and 95%, respectively, in milk samples with low somatic cell counts, similar to the values determined in this work.

***Cross specificity***. Table 3 summarises the logistic regression equation, detection limits of bioassay and MRLs for twenty-two ATB tested. The percentages of concordance coefficients were acceptable, between 79.5% (neomycin) to 96.2% (enrofloxacin), indicating an adequate fit with the logistic regression model. The $\beta_1$ coefficient values (comprised between 0.0002 for sulfadiazine and 0.0078 for oxytetracycline) presented an order of magnitude lower than those calculated for MC (Table 3), showing the low sensitivity of

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*B. megaterium* to detect other ATB, except for neomycin (below its MRLs) and tetracyclines (slightly higher than their MRLs).

Finally, this bioassay can be incorporated into microbiological systems in microtiter plates (MSmp) that classify residues of β-lactams, tetracyclines, sulfonamides and quinolones (Nagel *et al.* 2013) and thus increase the number of ATB groups to categorize. Indeed, the prior classification of ATB in the MC by the low-cost bioassay facilitates subsequent chromatographic analysis.

## CONCLUSIONS

To summarise, a bioassay containing *B. megaterium* spores provides detection levels near MRLs (Table 2) to classify MC frequently used for treating dairy cattle and its does so in a short response time (6 hours) compared with other microbiological methods in Petri dishes (16–18 h). Furthermore, a bioassay using spores instead of vegetative cells allows the production of microbiological test kits which could be preserved under refrigeration for commercialisation.

In addition, this bioassay could be used as a complementary analytical technique of available commercial screening test because it provides greater food safety of dairy products.

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