Development of an immunoassay for ciprofloxacin using phage-displayed antibody fragments

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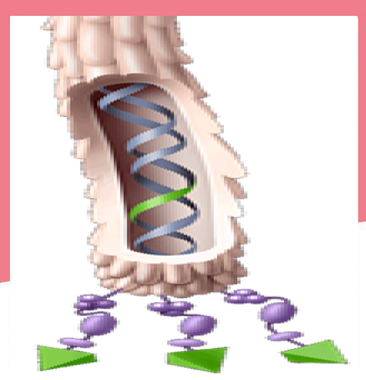

Introduction

Ciprofloxacin (CPFX), one of the most widely prescribed antibiotic [1], is a large spectrum synthetic drug of the fluoroquinolone's group. Because of its impact on health and environment, the EU has set a regulatory residual limit of 30 μg kg^{-1} for CPFX in edible animal tissues [2]. Therefore, ultra sensitive methods of detection and quantification need to be developed. CPFX is commonly determined by chromatographic procedures, namely HPLC and GC, but these techniques are laborious and time demanding. We report the isolation of phage-displayed antibody fragments (phAbs) against CPFX from a semi-synthetic phagemid library [3], as a step for the development of an immunoassay for CPFX.

Methods

1. CPFX immobilization on magnetic beads

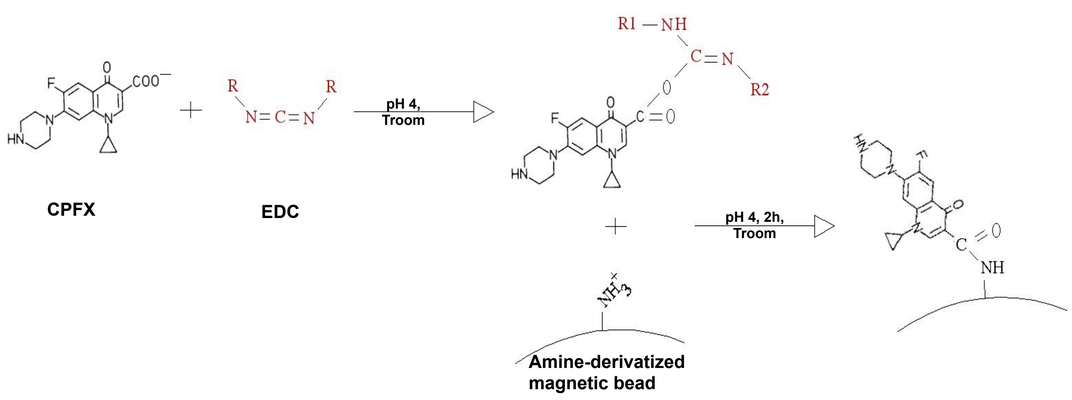

Figure 1 - Reaction scheme of immobilization of CPFX onto magnetic beads by the activation of carboxylic groups on CPFX with carbodiimide (EDC) and then the formation of an ester linkage to amine groups on magnetic beads

2. Selection by Phage Display

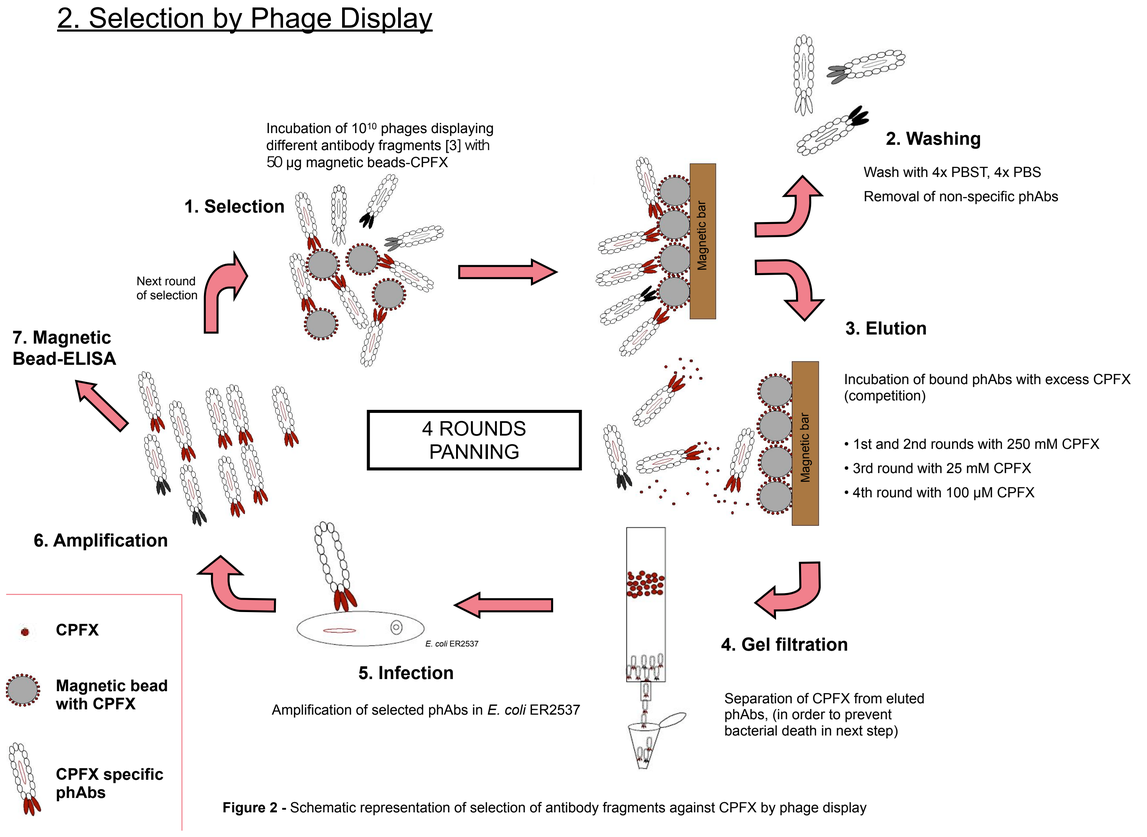

Figure 2 - Schematic representation of selection of antibody fragments against CPFX by phage display

3. Magnetic Beads-ELISA

In order to determine the content of CPFX-binding phAbs in samples, an ELISA using CPFX-magnetic beads was developed.

- Blocking of beads with soy proteins.
- Incubation of phAbs solution (approx,10^{10} phAbs) with 50 μg beads-CPFX, 2h, RT.
- Washing with PBST + PBS.
- Detection of bound phAbs using Anti-M13-HRP, 2h.
- Washing with PBST + PBS.
- Color development with ABTS solution for 30 min.
- Transfer to an ELISA plate and reading of OD at 405 nm.

Results

Table 1 - Results of immobilization assays. Percentage of CPFX unreacted was determined by HPLC-UV.

Ratio CPFX/Beads (μmol COO-/μmol NH3+)	% CPFX unreacted	% NH3+ reacted
0.5	10	53
0.9	9	82
1.1	16	94
1.3	**12**	**100**
2.5	58	100

CPFX was efficiently immobilized onto amine-derivatized magnetic beads using EDC (figure 1). Coupling efficiency was determined by quantification of unreacted CPFX by HPLC-UV.

Using a molar excess of 1.3x CPFX, all amine groups were bound to ciprofloxacin. Under these conditions 1 μg of beads suspension corresponds to about 2.5 nmol immobilized CPFX.

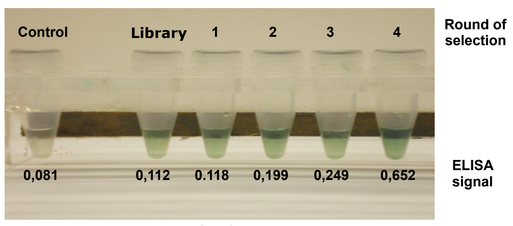

Figure 3 - Magnetic beads ELISA of polyclonal phAbs obtained from library to 4th round. Control was with any phAbs.

Enrichment of positive polyclonal phAbs against CPFX was confirmed by magnetic-beads ELISA. **ELISA signal increased after each round.**

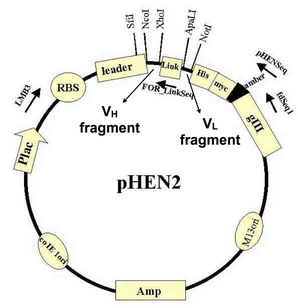

Figure 4 - Schematic representation of the phagemid pHEN2 from the synthetic library [3].

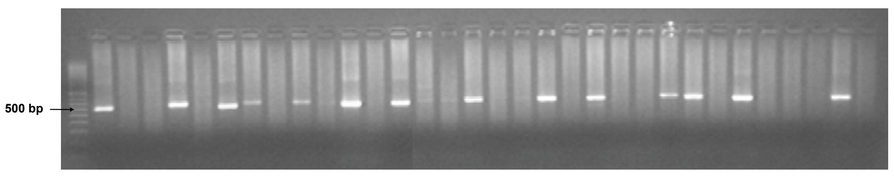

Figure 5 - PCR amplification using LMB3 and FdSeq1 primers of antibody fragment genes from individual ampicillin-resistant clones isolated from 4th round. The expected size of the PCR products was about 1000 bp (scFv). Interestingly, all are about 500 bp, suggesting display of a V_H fragment (See figure 4)

The PCR results indicate that all antibody fragments' genes amplified from individual clones obtained after the 4th round were **V_H genes.**

Eight PCR positive monoclonal phAbs were tested by ELISA in order to identify CPFX binding ones.

Six were positive to CPFX while 2 were negative. A control was performed with phAbs specific to one other ligand.

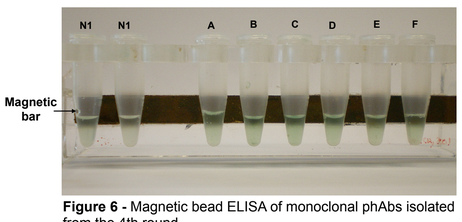

Figure 6 - Magnetic bead ELISA of monoclonal phAbs isolated from the 4th round

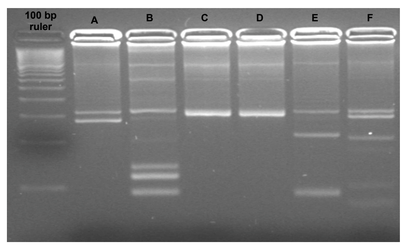

Figure 7 - RFLP analysis of PCR amplified antibody fragment genes

RFLP of individual amplified V_H fragments from the 4th round was performed by digestion with the BstNI restriction enzyme.

It was observed that there were **5 different phAbs**, while 2 were identical (clones C and D).

Conclusions

Five positive monoclonal antibodies-fragments against CPFX were efficiently isolated from a semi-synthetic phage antibody library by panning against immobilized CPFX on magnetic beads. PCR and RFLP analyses showed that isolated clones were V_H fragments. ELISA testing demonstrated affinity towards CPFX.

Future work

All five positive monoclonal phAbs will be tested to determine their ability to bind free CPFX (and other fluoroquinolones) by competitive elution. Selected ones will be used to produce soluble and purified V_H fragments. Their affinity constants will be estimated by competitive ELISA. Also, their ability to bind similar molecules will be evaluated. One or more V_H fragments will be used in immunoassays to determine and quantify CPFX on environmental samples.

[1] Ferech, M. et al., 2006. J. Antimicrob. Chemother. 58. 401-407
[2] Zonghui, Y. et al. 2001. Food Agr. Immunol. 13. 199-204
[3] Griffiths, A.D. et al., 1994. EMBO J. 13, 3245-3260