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Research paper

Development of an immunoassay for ciprofloxacin based on phage-displayed antibody fragments

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ABSTRACT

The widespread use of ciprofloxacin in human, animal and plant health has raised an environmental problem, paralleled by several other antibiotics. The aim of this work is the development of a rapid and sensitive ELISA assay for ciprofloxacin, which can constitute an alternative to time-consuming HPLC methods. For this purpose, we worked with antibody fragments, instead of whole antibodies, and used magnetic beads as solid support. Ciprofloxacin was successfully immobilized onto this support with a carbodiimide-mediated reaction. A library of phage particles that express human single-chain antibodies at their surface was then screened with an optimized protocol. Several positive fragments were isolated and identified as being V_L fragments. These were then fully characterized. A reproducible competitive ELISA was developed using the magnetic beads — ciprofloxacin as support and the phages displaying the V_L fragment as recognition entity. This assay showed limits of detection and quantification of 9.3 nM and 33 nM, respectively. Also, competitive ELISAs with ciprofloxacin homologues and other molecules showed cross-reactivities lower than 12%.

1. Introduction

Fluoroquinolones constitute a group of antibiotics with extensive utilization in human and animal health (Brown, 1996). This fact has led to concerns regarding the presence of antibiotic residues in food, groundwater and soils. Therefore, American, European, and Japanese authorities have established maximum limits for residues of the above compounds in several food items (U.S. Food and Drug Administration, 2005; European Union, 1990; Ministry of Health and Welfare, Japan, 2005).

The most common method for quantification of fluoroquinolones is high pressure liquid chromatography (HPLC), with several protocols published over the years (Ferdig et al., 2004; Krol et al., 1995; Manceau et al., 1999; Mehta et al., 1992; Tuerk et al., 2006; Yuan et al., 2001). All of the above are very laborious, time-consuming methods, and require sophisticated equipment. In many similar circumstances, an interesting alternative is immunoassays. Several of these assays have been developed for detection of drugs, pesticides and other products in different matrices. Immunoassays for fluoroquinolones, in particular ciprofloxacin (CPFX), have been reported (Duan and Yuan, 2001; Yuan et al., 2001). These assays were based on monoclonal antibodies, which are also quite difficult and expensive to produce.

This work aims the development of an immunoassay against CPFX using antibody fragments, namely single-chain variable fragments (scFvs). The selected source of antibody fragments is a phage library (Griffin.1 library) that expresses human scFvs as fusion proteins with the phage coat protein pIII (for a review, see Hoogenboom, 2005).

Selection from libraries of antibodies or antibody fragments with specificity for small haptens faces the difficulty of

Abbreviations: scFv, single-chain variable fragment;CPFX, ciprofloxacin; V_L, variable light chain.

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adsorbing these molecules onto solid surfaces. To overcome this problem, generally a preliminary step of conjugation of the small hapten to a carrier protein is needed. This procedure is cumbersome and brings difficulties in the selection process (Griffiths and Duncan, 1998), i.e., the common carrier proteins are also immunogenic and can lead to the selection of antibodies against the complex hapten–protein, instead of just the free hapten. To overcome these difficulties, we used paramagnetic beads with CPFX covalently bound to their surface and carried out the elutions with soluble CPFX. With these methods, we carried out the screening of the above phage library and arrived at a set of fragments with affinity for CPFX. Then, an extensive molecular and functional characterization of these fragments was carried out.

2. Materials and methods

2.1. Quantification of CPFX by HPLC

In order to quantify CPFX in solutions, an HPLC method with UV detection was used, based on the one described by Tuerk et al. (2006), but with modifications. A calibration curve was constructed using standard CPFX solutions (ciprofloxacin hydrochloride, AppliChem, Darmstadt, Germany) in the concentration range 10.0 μg/mL to 10.0 mg/mL, adjusted to pH 4.0. Samples were injected into a Zarobax SB-C18 column (Agilent, Santa Clara, CA, USA), mounted on a Beckman System Gold (Palo Alto, CA, USA), equipped with an autosampler 508, pumps 126, and an UV–VIS 162 detector module, set at 280 nm wavelength. The mobile phase comprised: solvent A: aqueous 0.1% formic acid (Fluka, Lisbon, Portugal); and solvent B: 0.1% formic acid in acetonitrile (Merck, Lisbon, Portugal). The elution gradient was: 0–1 min: 90% A; 1–22 min: 30% A; 22–28 min: 90% A. The flow rate was 0.3 mL/min. The reproducibility of this protocol, as well as the limits of detection and quantification, was determined by the reported methods (Silva Ferreira et al., 2003).

2.2. Immobilization of CPFX onto magnetic beads

For the isolation of specific antibody fragments, CPFX was first coupled to magnetic beads. For this purpose, 100 μL of MagnaBind Amine-Derivatized Beads (Pierce, Rockford, IL, USA) were transferred to an Eppendorf tube (previously weighed) and washed twice with 1 M NaCl solution. After removing the supernatant from the last washing, the beads were weighed. They were then suspended in 400 μL of aqueous solution containing 4.5 mg of CPFX, with pH adjusted to 4.0. N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, Biochemika, Milwaukee, WI, USA), 10 mg, were dissolved in 100 μL of water and this solution was immediately added to the beads suspension. The reaction volume was adjusted to 1 mL and the pH adjusted to 4.0. Reaction was allowed to proceed for 2 h, at 25 °C and with gentle agitation. After that, the coupling solution was removed and the beads were washed twice with 1 M NaCl. Coupling yield was determined by HPLC.

The above protocol corresponds to the standard procedure. In an initial phase of the project, an optimization of this procedure was carried out. For that purpose, the amount of CPFX in the binding solutions was varied between 1 and 4.5 mg. Also, control reactions were carried out in a similar way, but without magnetic beads.

2.3. Selection cycles of phage display

The source of the antibody fragments was the Griffin.1 library which is composed of human scFvs containing highly diverse CDR3s in both the V_H and V_L (Griffiths et al., 1994). This library was derived by recloning human V_H and V_L fragments into the phagemid vector pHEN2. ScFvs can be displayed on the surface of bacteriophage when expressed in suppressor *Escherichia coli* strains (e.g., ER2537, TG1) or as soluble fragments that also contain the c-*myc* tag and carboxyl-terminus His-tag in nonsuppressor *E. coli* strains (e.g., HB2151).

Selections from Griffin.1 library of phage particles displaying scFv fragments were performed on magnetic beads with covalently coupled CPFX. Approximately 10^{12} phage particles from the library were added to a sample of beads with *ca.* 40 μg of coupled CPFX, suspended in 1 mL of PBS containing 1% of soy protein. The reaction proceeded for 90 min at room temperature (RT), with constant agitation. The beads were then washed 6× with PBST (PBS containing 0.1% tween-20) and 6 times with PBS, alternatively, in order to minimize non-specific binding. In each washing step, the contact time between beads and solution was 1 min, followed by 1 min of magnetic sedimentation. The phage particles were then specifically eluted by incubation for 30 min, at RT and with constant agitation, with a solution of 25 mM CPFX. The beads were separated from this solution and the supernatant was collected.

Since CPFX is an antibiotic, and the next operation is the infection of *E. coli* with the eluted phages, an additional step of removal of CPFX from that solution is needed. The eluted phages were separated from CPFX using a 10 mL Econo Pac 10DG desalting column (Bio-Rad Laboratories, Munich, Germany). The efficacy of this procedure was tested previously, with good results. The phages are eluted in the void volume and with no substantial losses, whereas CPFX is eluted at considerably higher volumes. The phage suspension, free of CPFX, was then used to infect exponentially growing *E. coli* ER2537 cells for 30 min, at 37 °C. The cells were then spread on large "Nunc Bio-Assay Dishes" (Nunc, Langenselbold, Germany) containing TYE with ampicillin (100 μg/mL) and 2% glucose, and these dishes were incubated overnight at 37 °C. Next day, cells were scraped from the plate and grown on 2× TY broth containing ampicillin (100 μg/mL) and 2% glucose until the OD600 reached 0.5. Phage particles were rescued with M13KO7 helper phage, using a ratio of cells: helper phage of 1/20, and incubating for 30 min at 37 °C, without agitation. The suspension was centrifuged at 3300 *g* for 10 min at 4 °C, the cells resuspended on 2× TY containing ampicillin (100 μg/mL) and kanamycin (25 μg/mL), and incubated overnight at 30 °C in order to produce phage antibodies. Next day, phage particles were precipitated using PEG/NaCl, as described elsewhere (Barbas et al., 2001).

The subsequent selection cycles were identical to the one described, except for the concentration of CPFX in the eluent and the incubation time: on the second cycle, 25 mM CPFX for 20 min; on the third cycle, 2.5 mM CPFX for 15 min; and on

the fourth cycle, 100 µM CPFX for 10 min. In order to evaluate the positive enrichment against CPFX, a binding ELISA was performed with the polyclonal phages obtained after each cycle.

2.4. Polyclonal phage ELISA

A sample of magnetic beads suspension containing *ca.* 40 µg immobilized CPFX and another similar sample of beads without CPFX (control) were blocked with 2% soy protein for 2 h, at RT, with constant agitation. The beads were washed once with PBS and then *ca.* 10^{10} polyclonal phage particles from each selection cycle were added to the beads, suspended in 1 mL PBS containing 1% of soy protein. Incubation took place for 2 h at RT, with constant agitation. The beads were then washed 3× with PBS and 3× with PBST, as described before. Next, 200 µL of anti-M13-HRP conjugated antibody (Roche, Mannheim, Germany), diluted 5000-fold, were added to the beads, and incubated for 90 min at RT, with constant agitation. The beads were washed with 3× PBS and 3× PBST. The colorimetric reactions were promoted with 200 µL ABTS solution (0.01% ABTS [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)], 0.01% H_2O_2 in 0.1 M citrate buffer, pH 4) for 30 min. The solutions were separated from the beads and transferred to a 96-well microplate, in order to measure their OD405 in a plate reader (model 680, Bio-Rad UK, Herts, UK).

2.5. Screening for positive clones

Clones obtained after the last selection cycle were first screened for the presence of scFvs. The presence of insert on those clones, as well as its size, was evaluated by PCR amplification of the scFv segment. For these purposes, their DNA was excised from the cells by boiling a colony in 50 µL ddH_2O for 10 min, centrifuging for 10 min at 13,000 *g* (microcentrifuge Eppendorf 5415D, Hamburg, Germany) and using the supernatant for PCR. The fragments were then amplified using the primers LMB3 (5′-CAGGAAACAGC-TATGAC-3′) and FdSEQ1 (5′-GAATTTTCTGTATGAGG-3′), as described by Griffiths et al. (1994). The PCR product was then analyzed by electrophoresis on 1% agarose gel.

2.6. Monoclonal phage ELISA

This phage ELISA was performed similarly to the polyclonal phage ELISA, except that the phage particles tested were the ones rescued individually from the clones that showed presence of insert by PCR analysis.

2.7. DNA sequencing

Plasmid DNA from the selected clones was purified using MiniPrep kit (QIAGEN, Hilden, Sweden). Next, amplification was done using LMB3 primer and the sequencing kit BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. The products were analyzed on an automatic sequencer ABI3730 (Applied Biosystems, Foster City, CA, USA). After translating to amino acid sequences, they were analyzed on VBASE database (Tomlinson et al., 1995). Their CDRs were classified according to the definition by Kabat et al. (1991).

2.8. Competitive ELISA

Competitive ELISA was performed essentially as the previous ELISAs, using magnetic beads with covalently coupled CPFX, except that the monoclonal phage particles were incubated with the beads in the presence of varying concentrations of soluble CPFX (from 4.3 nM to 2.7 µM). The percentage of binding in each case was estimated by the ratio between the signals obtained in the presence and in the absence of competition in solution. The limits of detection (LOD) and quantification (LOQ) of this ELISA were defined as three times and ten times the standard deviation of the lowest detectable CPFX concentration, respectively (Duan and Yuan, 2001).

The specificity of the clones were analyzed by competitive ELISA with three CPFX analogues (enrofloxacin, ofloxacin and norfloxacin), and with another two unrelated antibiotics (penicillin and gentamicin), at three different concentrations. The cross-reactivity was quantified by the ratio of the molar amount of CPFX for 50% binding to the molar amount of competitor species for 50% binding (Duan and Yuan, 2001).

3. Results and discussion

Immunoassays against small haptens are hindered by the difficulty of adsorbing them onto solid supports. Commonly, a preliminary, laborious step of conjugating the hapten with a carrier protein — which mediates the adsorption to the support — needs to be carried out (Duan and Yuan, 2001; Watanabe et al., 2002). Alternatively, the small ligand might be covalently bound to the solid support, such as ELISA plates. However, difficulties in developing such assays with plates have been reported (Kala et al., 1997), and also observed in our work. An interesting alternative to plates is the utilization of paramagnetic beads as support. For this purpose, we targeted to bind the free carboxylic group in the CPFX molecule to amine-derivatized beads via a carbodiimide reaction. Table 1 summarizes the results of several immobilization experiments, where information regarding amounts of amine groups in beads, CPFX in binding solutions, and CPFX attached to the beads is included. The quantities of amine groups were estimated based on manufacturer's information (240 µmol amine groups per gram of beads). In experiments 1 and 2, we had initially more amine groups than CPFX. Close to 90% of the CPFX originally present in solution was bound. In experiments 3 to 5, we had more CPFX than amine groups. An excess of *ca.* 30% was sufficient to saturate the amine groups.

Table 1
Immobilization of CPFX on to magnetic beads: initial conditions, CPFX bound and percentage saturation of amine groups.

Experiment	Initial CPFX (µmol)	Initial NH_3^+ (µmol)	Initial molar ratio CPFX/NH_3^+	CPFX bound (µmol)	Saturation of NH_3^+ (%)
1	2.27	3.12	0.73	2.04	65.4
2	3.40	3.83	0.89	3.11	80.5
3	5.67	5.16	1.10	4.78	92.6
4	7.93	6.23	1.27	6.98	112
5	10.19	4.03	2.53	4.23	105

Table 2
Experimental conditions of the various phage selection cycles and their final recoveries.

Cycle	Incubation time (h)	Washings	[CPFX] for elution (mM)	Elution time (min)	Recovery (%)
1	2	4×PBST+ 4×PBS	25	30	2×10^{-3}
2	2	4×PBST+ 4×PBS	25	20	7×10^{-6}
3	1.5	8×PBST+ 8×PBS	2.5	15	1×10^{-5}
4	1	10×PBST+ 10×PBS	0.10	10	3×10^{-4}

Note that saturation percentages above 100% result from the method of estimating quantities of amine groups. Quantification of CPFX in the binding solutions of control reactions showed that no more than 2.2% of the initial amounts were lost. This indicates that no significant side reactions occurred. Therefore, very efficient binding of CPFX to the amine groups was achieved, even without the presence of a coupling enhancer, such as N-hydroxysulfosuccinimide (Ivanova et al., 2006; Riedstra et al., 1998).

For the isolation of antibody fragments specific for CPFX, we used the human semi-synthetic Griffin.1 library, which consists of a phage-displayed library of high diversity (Griffiths et al., 1994). Phage particles binding to CPFX were isolated with four rounds of selection, using the conditions described in Table 2. Along successive cycles, the washing and elution conditions were changed. On the first two cycles, the washings were less stringent and the elution was carried out with higher concentrations of free CPFX, in order to avoid loss of positive clones, especially the low affinity ones. The elution time also decreased with the cycles. The percentage of recovery of phage particles in each cycle, which is based on the ratio between their final and initial numbers, was monitored. This number decreased from the first cycle to the second, which might be due to the very high diversity of the initial library, in comparison to the diversity after the first round. The recovery then increased in the posterior rounds, suggesting that specific antibody fragments were being selected along the cycles.

The enrichment along the cycles was confirmed by polyclonal ELISA. On a first attempt, we tried to covalently bind CPFX to 96-well plates displaying amino groups on the surface, by a method similar to the one used with magnetic beads. The results (data not shown) lead to no detection of positive binding, even though several conditions for coupling of CPFX and several ELISA protocols were tested. We then changed to the magnetic beads. Using this support, we observed positive binding, and the ELISA signals increased along the selection cycles (Table 3). Similar results have been observed previously by Kala et al. (1997). These authors registered an enrichment of phage titers along the selection cycles against alkaline phosphatase, but they could not detect positive phage by conventional ELISA in 96-well plates; however, they did obtain positive results using magnetic beads coupled with the target antigen. Two possible explanations for these facts can be the higher quantity of immobilized ligand in the beads and its more favorable spatial arrangement, compared to the ELISA plates.

Table 3
Signals (OD_{405}) in ELISAs with polyclonal phage particles from the various selection cycles. Values are means of triplicates and the standard deviations were less than 10%. As negative control, a monoclonal phage-displayed scFv fragment specific to an irrelevant protein was used.

Average signal (OD_{405})	Negative control	Griffin Library	Cycle 1	Cycle 2	Cycle 3	Cycle 4
Beads with CPFX	0.059	0.112	0.118	0.199	0.249	0.625
Beads without CPFX	0.064	0.051	0.062	0.049	0.053	0.067

Table 4
Signals (OD_{405}) in ELISA of monoclonal phage particles obtained after the fourth cycle of selection. Values are means of triplicates and the standard deviations were less than 10%. A monoclonal phage-displayed scFv fragment, specific to an irrelevant protein, was used as negative control. A clone is classified as positive (+) to CPFX if its signal is higher than three times its control signal (beads without CPFX), and negative (−) otherwise.

Average signal ($OD_{405\,nm}$)	Negative control	Clone 404	Clone 406	Clone 411	Clone 419	Clone 407	Clone 410
Beads with CPFX	0.028	0.184	0.384	0.354	0.194	0.041	0.050
Beads without CPFX	0.035	0.051	0.048	0.039	0.057	0.047	0.055
		+	+	+	+	−	−

After confirming the enrichment, clones from the last cycle were then plated out and picked for screening. The presence of insert was first monitored by PCR amplification of the insert. Among 32 clones screened, only 17 contained a fragment (or could be amplified). The lack of insert is a relatively common event and it has been studied before (Berdichevsky et al., 1999). Basically, clones that lose the insert accumulate along the selection cycles because they have a growth advantage (their plasmid is shortened). Furthermore, electrophoresis with the clones containing inserts revealed that all those 17 inserts had just half of the size of a scFv, meaning that only one domain (either V_H or V_L) was present.

Six out of those 17 clones were chosen for further studies. Their phages were rescued and screened individually for binding CPFX coupled to magnetic beads, in ELISA. Four out of the six phage-displayed fragments (404, 406, 411 and 419) showed positive signals (Table 4).

The four positive clones were then sequenced and the fragments were classified according to the VBASE database (Tomlinson et al., 1995). All four were V_L fragments of the V family (Table 5). Clone 411 had the same sequence as the 406 one on the DNA level.

In order to evaluate the ability of each phage-displayed clone to bind the soluble form of CPFX, and not only CPFX coupled to the beads, a competitive ELISA was carried out. In

Table 5
Characterization of selected fragments according to the VBASE database.

Fragment	Family	Segment	CDR-L3
404	VκI	DPκ9	TQATQFRT
406/411	VκII	DPκ17	QQSYSTHT
419	VκII	DPκ15	MQALQTLWIT

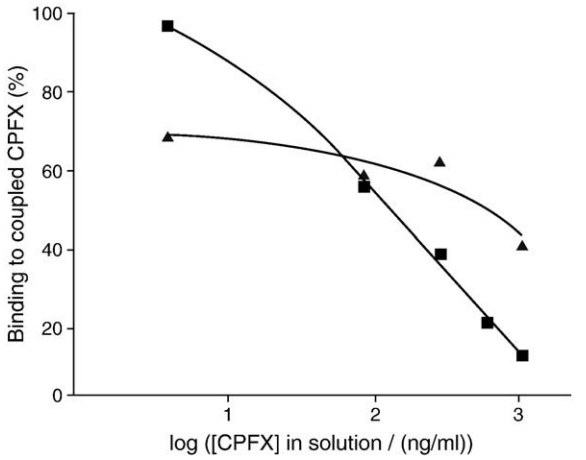

Fig. 1. Competitive ELISA with soluble CPFX for selected phage-displayed antibody fragments (■: clone 406; ▲: clone 419). The horizontal axis represents the concentration in logarithmic scale of soluble CPFX, which competes with bound CPFX. The vertical axis represents the fraction of phages bound to the beads in each equilibrium situation.

this assay, an increasing amount of CPFX in solution competes with bound CPFX for phage binding. One can observe from Fig. 1 that, for clones 406 and 419, soluble CPFX displaces the phages from the beads. This indicates that these clones are indeed specific to CPFX and are able to bind to the free form of the molecule. The competitive effect of free CPFX is more pronounced in clone 406 than in 419, suggesting that the former might have higher affinity for CPFX in solution, being then more suitable for set-up of an immunoassay. Therefore, clone 406 was chosen for further analysis. Clone 404 could not be displaced by free CPFX (data not shown).

The limits of detection and quantification of CPFX of this ELISA with the clone 406 were estimated at 9.3 nM and 33 nM, respectively. The standard deviations on both inter and intra assays were less than 5%, in all experiments. Reported HPLC methods for quantifying CPFX (Krol et al., 1995; Manceau et al., 1999) have limits that, at the best, are of the same order of magnitude as the above, but they can be up to 10 times higher. Our HPLC method has limits of detection and quantification of 66 nM and 98 nM. However, different ELISAs for CPFX reported in the literature (Duan and Yuan, 2001; Watanabe et al., 2002) were more sensitive than the one developed here, ranging from 1 nM to 10 nM. The likely reason for these higher sensitivities is that the antibodies used on those ELISAs were obtained after animal immunization with CPFX. This allows a natural affinity maturation of the antibodies against the target. On the other hand, the library used in this work is a non-immunized library, so it is unlikely to have the presence of fragments with comparable affinities. However, in order to increase affinity, the fragment we selected can still be subjected to an affinity maturation process.

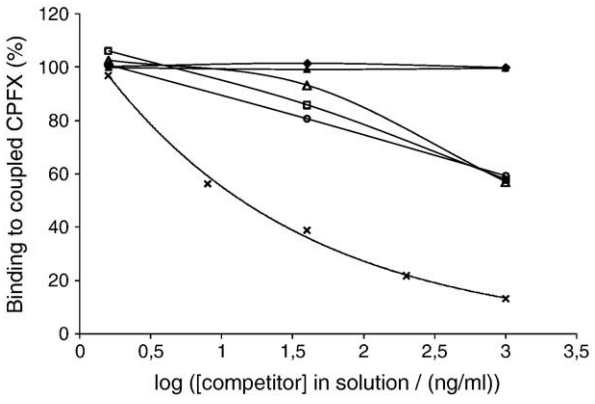

Fig. 2. Competitive ELISA using the phage-displayed clone 406 and different competitors in solution. The horizontal axis represents the concentration in logarithmic scale of competitor in solution (▲: penicillin; ♦: gentamicin; □: norfloxacin; Δ: enrofloxacin, ○: ofloxacin; x: CPFX), which competes with magnetic beads-bound CPFX. The vertical axis represents the fraction of phages bound to the beads in each equilibrium situation.

Table 6
Cross-reactivities in competitive ELISA of the phage-displayed fragment from clone 406 with fluoroquinolones and with two unrelated antibiotics. Values are means of triplicates and the standards deviations are less than 10%.

	Cross-reactivity (%)
CPFX	100
Ofloxacin	5.2
Norfloxacin	8.3
Enrofloxacin	12.3
Penicillin	<0.01
Gentamicin	<0.01

The cross-reactivity of the fragment 406 with other molecules was also tested. For this purpose, a competitive ELISAs using three CPFX analogues (of the fluoroquinolone family, namely, ofloxacin, norfloxacin and enrofloxacin) and two non-related antibiotics (penicillin and gentamicin) were carried out. Penicillin and gentamicin did not compete out the phage-displayed V_L fragment from bound CPFX (Fig. 2). On the other hand, the three CPFX analogues can compete with CPFX for the fragment, but with lower affinities. This is not surprising, since their structures are very similar. The cross-reactivities between the CPFX analogues and CPFX were quantified (Table 6). Enrofloxacin is the one with higher cross-reactivity (12%), and is also the one with a structure more similar to CPFX. Ofloxacin is the less similar to CPFX and showed the lower cross-reactivity.

4. Conclusions

The aims of this work were to isolate antibody fragments specific to CPFX and to develop a rapid and sensitive method for detecting and quantifying CPFX. For this purpose, the phage-display, semi-synthetic Griffin.1 antibody fragments library was used. Four selection cycles were performed against CPFX coupled to magnetic beads and using an optimized washing and elution protocol. Among a relatively small number of screened clones, three specific V_L fragments were obtained, all belonging to the Vκ family. In order to evaluate the specificity and affinity of these fragments, a protocol of phage ELISA using the CPFX-derivatized magnetic beads was developed. Two clones were competed out by free CPFX. Using the clone with best results, the limits of detection and quantification of the competitive ELISA were below the threshold limits of CPFX in diverse samples (Golet et al., 2001;

Duan and Yuan, 2001). This suggests the potential of the selected fragment for quantitative assays against CPFX. Also, that fragment is quite specific for CPFX, showing less than 12% of cross-reactivity with structurally similar fluoroquinolones, and no cross-reactivity with two unrelated molecules.

These data were obtained with the fragment displayed on the surface of the phage, showing that this format can be suitable for the detection and quantification of CPFX in an aqueous solution. In fact, phage-displayed antibody fragments have been reported to be a good substitute to the isolated fragments (Petrenko and Smith, 2000; Petrenko, 2008) and, in many cases, might even be more convenient. Notwithstanding, future work can include the production at medium scale of the isolated fragment, and the development of ELISA tests with them, similar to the tests reported here.

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