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A robust high-throughput fungal biosensor assay for the detection of estrogen activity

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ABSTRACT

Estrogenic active compounds are present in a variety of sources and may alter biological functions in vertebrates. Therefore, it is crucial to develop innovative analytical systems that allow us to screen a broad spectrum of matrices and deliver fast and reliable results. We present the adaptation and validation of a fungal biosensor for the detection of estrogen activity in cow derived samples and tested the clinical applicability for pregnancy diagnosis in 140 mares and 120 cows. As biosensor we used a previously engineered genetically modified strain of the filamentous fungus *Aspergillus nidulans*, which contains the human estrogen receptor alpha and a reporter construct, in which β-galactosidase gene expression is controlled by an estrogen-responsive-element. The estrogen response of the fungal biosensor was validated with blood, urine, feces, milk and saliva. All matrices were screened for estrogenic activity prior to and after chemical extraction and the results were compared to an enzyme immunoassay (EIA). The biosensor showed consistent results in milk, urine and feces, which were comparable to those of the EIA. In contrast to the EIA, no sample pre-treatment by chemical extraction was needed. For 17β-estradiol, the biosensor showed a limit of detection of 1 ng/L. The validation of the biosensor for pregnancy diagnosis revealed a specificity of 100% and a sensitivity of more than 97%. In conclusion, we developed and validated a highly robust fungal biosensor for detection of estrogen activity, which is highly sensitive and economic as it allows analyzing in high-throughput formats without the necessity for organic solvents.

1. Introduction

Naturally occurring estrogens are cholesterol-derived members of the steroid hormone family, which are important for sexual development of vertebrates and play an essential role in the female reproductive cycle [1–3]. The class of estrogens includes compounds, such as estrone, estradiol and estriol, which share the same chemical backbone structure [4]. Besides their physiological function in the reproductive cycle, estrogens have also been associated with the development of breast, ovarian and prostate cancer, and reproductive abnormalities in vertebrates [5–7]. The genotoxic effect has been linked to long term exposure to elevated estrogen levels, which leads to accumulation of genotoxic catechol metabolites derived from estrogen metabolism [8–10]. Prolonged exposure may be linked to consumption of food containing high levels of natural estrogens, for example milk or food contaminated with estrogen mimicking compounds, so called xenoestrogens [11–14]. Xenoestrogens are for example derived from plants (phytoestrogens) or packaging plastic materials [14]. Estrogenic active compounds or endocrine disruptors may alter the natural hormone cycle by modifying regulatory pathways, competing for the binding of estrogen receptors, antagonizing endogenous estrogens or mimicking estrogen dependent effects [11,15]. Thus, the determination of natural estrogen and xenoestrogen levels in potential sources of estrogenic active compounds is an important task.

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The gold-standard are methods based on the use of either gas chromatography/mass spectrometry or multi-residue-liquid chromatography tandem mass spectrometry, which are able to detect small amounts of estrogens in a variety of sample matrices. However, the sample matrices have to undergo extensive chemical extraction protocols prior to measurements and also the maintenance of costly infrastructure is required [16–18]. Methods based on antibodies are less expensive, however, equally susceptible to matrix effects and thus, the samples need to be extracted prior to measurement [19]. Another type of estrogen detection methods are receptor based bioassays, which have been realized in human cell lines, bacterial cells and yeast cells [20–26]. Receptor based bioassays are useful to detect all compounds, which are able to penetrate living cells and have affinity for a given receptor. In addition, assays based on human cell lines would give advantages over other systems due to their high sensitivity and ability to identify estrogenic compounds that require human metabolism for activation [27]. However, human cell lines are not robust and require delicate handling compared to yeast or bacterial cells. Systems based on yeast or bacteria strains give the advantage of robust cell lines, low maintenance costs, media devoid of steroids and the lack of known endogenous estrogen receptors [28]. Thus, yeast strain based assays have been extensively used for the detection of estrogenic compounds in water, urine of calves, food packaging, soil and surfactants [11,28–31]. However, all these assays still need chemical sample extraction prior to measurement, which leads to organic solvent and toxic compound waste.

Thus, to reduce the usage of organic solvents and toxic compounds the search for estrogen-reporting cell lines able to cope with non-extracted samples or reduced extraction protocols but at the same time still displaying high sensitivity and specificity for estrogenic compounds is essential. The estrogen-responsive expression system developed in our group as metabolically-independent gene expression tool uses the highly robust filamentous fungus *Aspergillus nidulans* [32], which belongs to a fungal group known to be strongly competitive and persistent in nature enduring various detrimental environments.

Beside the detection of natural estrogens or xenoestrogens as described above, the use of estrogen detection tests is of interest to livestock industry. Evaluation of the natural estrogen levels in livestock may be useful for the detection of pregnancy, estrus or reproductive abnormalities, for example cryptorchidism in horses [33–35]. It has been shown that the estrogen level in milk and urine of cows correlates with the level in blood [36]. Previous studies have demonstrated that fecal estrogen levels can be used for pregnancy diagnosis in cows [34,37] and mares with breed dependent differences in the estrogen content [35]. In cows, 17α-estradiol is the major excreted estrogen metabolite [38], and equilin and equilenin are typical equine estrogen metabolites. These estrogens are synthesized by the placenta during pregnancy and can therefore be used for pregnancy diagnosis [37,39]. In large scale cattle farming it is important to use easy to obtain and non-invasive matrices, such as milk or feces for estrogen detection. High-throughput methods for the detection of estrogens in cows and horses have received increasing clinical interest [40,41]. Therefore, it is imperative to develop innovative analytical methods that are able to handle non-invasive matrices and deliver fast and reliable results.

The objective of this study was to assess the applicability of the estrogen-responsive fungal expression system as a robust alternative to other bioassays or biosensors and to evaluate the possibility of this system to tolerate non-extracted sample matrices. In this study, we used samples taken from cows including saliva, blood, milk, urine and feces. Furthermore, the clinical applicability of the fungal biosensor assay for pregnancy diagnosis in cows and mares was assessed using feces as non- or minimal-invasive sampling material.

2. Experimental

The study was approved by the institutional ethics committee and the national authority according to § 26 of Animal Experiments Act (Tierversuchsgesetz-TVG 2012) 68.205/0190-WF/V/3b/2014).

2.1. Fungal bioassay

2.1.1. Chemicals

The compounds estrone, 17β-estradiol, 17α-estradiol, estrone sulfate and estrone glucuronide were purchased from Sigma Aldrich (Germany). All compounds were dissolved in ethanol (100 μg/mL) and stored at −20 °C. Ammonium tartrate, p-amino benzoic acid, biotin and riboflavin were obtained from VWR (Germany). ONPG (o-nitrophenyl-β-D-galactopyranoside) was obtained from ThermoFisher scientific (Germany) and dissolved in sterile water at a concentration of 5 mg/mL. β-D-Glucuronide glucuronosohydrolase was obtained from Sigma Aldrich (Germany). 5-Brom-4-chlor-3-indoxyl-β-D-Galactosid (X-Gal) was obtained from Roth (Germany).

2.1.2. Strain

The *Aspergillus nidulans* strain (ERE-URA-RS; *riboA1*) [32] was taken from our strain collection and inoculated as described on *Aspergillus* minimal medium agar (AMM) supplemented with appropriate amounts of riboflavin, p-amino benzoic acid and biotin according to standard protocols [32]. The bioreporter strain harbors two expression cassettes stably integrated in the genome. One contains the human estrogen receptor alpha (*hERα*) driven by a constitutive fungal promoter and the second one contains the β-galactosidase reporter driven by an estrogen-responsive element (ERE) containing promoter construct. The promoter architecture provides a very strong transcriptional response to the presence of estrogens and can thus be regarded as highly sensitive to estrogenic compounds [32].

2.1.3. Sample preparation and fungal biosensor assay

The sample matrices were diluted 1:1 (v/v) with 96% ethanol. Fecal samples were diluted 1:1 (w/v) with 96% ethanol. Prior to measurement, 3 μL of the biosensor strain were inoculated at a concentration of 10^6 spores/mL on supplemented AMM agar (1% glucose, KCL, KH_2PO_4, K_2HPO_4,$MgSO_4*7H_2O$, Hutner's trace elements [42] and 1.5% agar) and incubated at 37 °C until a visible white culture spot had developed (roughly 14–16 h). Samples and standards were dissolved in 96% ethanol and pipetted (5 μL) directly on top of the fungal culture spots. The fungal biosensor strain was further incubated at 37 °C for 6 h to induce the reporter. To perform qualitative estrogen activity screening 3 μL of X-Gal were pipetted directly onto the fungal culture spots and visible color development was noted as early as 30 min after X-gal addition (strong activity) up to 4 h after X-Gal addition (weak activity). Incubation during X-Gal staining was proceeded at room temperature. The limit of detection was estimated using standards. The limit of detection was calculated by using the standard deviation of the reference value of water and ethanol by adding three times the standard deviation on the mean value of the water and ethanol reference.

For a semi-quantitative determination of estrogenic activity in relation to the standards, the incubated fungal culture spots were stamped out together with the underlying agar. The agar stamp was transferred into a 96 deep well plate containing lysing matrix Z (2.0 mm Yttria-Stabilized Grinding Beads) from MP Biomedicals (Germany) and 750 μL of 100 mM buffer Z ($Na_2HPO_4*H_2O$, KCl and $MgSO_4*7H_2O$). The cell lysis was performed in a FastPrep-96 from MP Biomedicals (Germany) three times for 30 s at 1800 rpm. After lysis the plates were centrifuged at 4 °C for 20 min at 4000*g* to pellet cell debris and agar traces. From the supernatant, 100 μL were transferred into a novel 96 shallow well plate and a Bradford protein assay (Bio-Rad, Germany) was performed to normalize the β-galactosidase activity to protein content of the induced and control culture spots. Furthermore, 100 μL of the supernatant were transferred into a novel 96 shallow well plate and mixed with 100 μL of buffer Z and 20 μL of ONPG (5 mg/mL). After a yellow color developed in the standards, the reaction was stopped by adding