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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

10 µL of 1 M $NaCO_3$ solution. The reaction was measured in a multi plate reader (Synergy H1, Biotek, USA) at absorbance of 420 nm and 610 nm for background deduction. As negative controls fungal culture spots not confronted with anything, culture spots confronted with solvent only (3 µL of 96% ethanol) and culture spots confronted with 5 µL of water were used.

The high-throughput assay was performed on Hamilton Starlet platforms (Switzerland) as described above with the adjustment that the fungal spore solution was pipetted into 96 deep well plates (Nerbe, Germany) containing the lysing matrix Z with an overlay of modified AMM (1% agar content). After incubation, 5 µL of sample and standard were pipetted automatically onto the fungal culture spots followed by incubation of the plates for 6 h at 37 °C. Lysis was performed in the FastPrep 96 (MP Biomedicals, Germany) followed by centrifugation at 4000*g*. The supernatant was transferred into 96 shallow well plates (Corning, Germany).

2.2. Determination of assay characteristics

2.2.1. Animals and experimental design

All matrices derived from cows used for assay characterization and validation were collected during this study. The sample collection was performed from March to April 2015 on the Teaching and Research Farm Kremesberg, University of Veterinary Medicine, Vienna, Austria. The animals were housed in freestall barns with straw-bedded cubicles. From six cows, plasma, feces, urine, saliva and milk were taken at 7 different time points. The estrous cycle of each cow was synchronized using the previously described OvSynch program [43]. In brief, animals received Gonadotropin-Releasing-Hormone (GnRH)-analogon buserelin (0.01 mg, i.m., Receptal, MSD-Intervet, Austria), seven days later Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-analogon cloprostenol (0.5 mg, i.m., Estrumate, MSD-Intervet, Austria) and two days after $PGF_{2\alpha}$, a second dose of GnRH was administered. All six cows ovulated between 24 and 36 h after the last GnRH injection, which was confirmed by transrectal ultrasonographic examination using a 5 MHz linear-array transducer (Easy-Scan, BCF Technology, Scotland). Six sampling time points were chosen around ovulation (at the time point of the last GnRH injection and 6, 12, 24, 36 and 48 h later). One sampling time point was during diestrus, 14 days after the last GnRH injection. These time points were chosen to obtain samples with estradiol concentrations in the physiological range of the reproductive cycle [44]. Blood was taken using an S-Monovette (Sarstedt, Germany) and lithium heparin as anticoagulant, and centrifuged at 2000*g* for 10 min to obtain plasma. Fecal samples were collected directly from the rectum with a gloved hand, and urine collection was performed by manual stimulation of the ventral vulva commissure. Saliva was collected using Salivettes (Sarstedt, Germany) followed by centrifugation at 1000*g* for 10 min. Plasma, feces, urine and saliva samples were stored at −20 °C until further analysis. Milk samples were taken during the daily milking routine. Pre-milk was gained by hand before the mechanical milking, main-milk was sampled from the collector of the milk machine and post-milk was manually collected after the milking process. The milk was either stored at −20 °C until analyses by an enzyme immunoassay or at 4 °C for analysis with the fungal biosensor assay. Additionally, 10 representative samples from the daily diet of the cows were taken and analyzed for estrogen activity.

2.2.2. Pregnancy tests in fecal samples of mares and cows

To evaluate the fungal biosensor assay for a clinical application, it was used to assess the estrogen level in mare and cows fecal samples to determine or exclude pregnancy. Mares fecal samples are routinely sent to the laboratory of our partner for pregnancy determination. The fecal samples are routinely tested by the established estrogen EIA test [35]. A total number of 140 mare fecal samples, sent in by their respective owners, were used for the bioassay and compared to the results obtained with the EIA test. In addition, 120 fecal samples were taken from pregnant cows at 4 different gestation periods (each n = 30), i.e. 40–60, 100–120, 180–200, 260–280 days and from 22 not pregnant cows. The estrogen activity was determined with the fungal biosensor assay and the results were compared to clinical examinations.

Using the estrogen activity of both sets of samples the cut off value for pregnancy was established.

2.2.3. Decision limit and detection capability

The fungal biosensor was validated with ethanol solved estrogens to assess specificity and sensitivity for the substrates. Thus, dilutions of 17β-estradiol, the main active estrogen in the reproductive cycle, in ethanol (0.01 ng/L to 100 mg/L) were used. Furthermore, estrone, 17α-estradiol, estrone sulfate and estrone glucuronide were tested.

17β-estradiol was used as standard for the evaluation of the matrix samples. The results were therefore considered as 17β-estradiol equivalents.

The fungal biosensor assay was validated for the matrices bovine plasma, feces, urine, saliva and milk. Samples from 6 cows at 8 time points per matrix (n = 400 including replicates) were spiked with 17β-estradiol (15 and 1.5 ng/L). In order to determine potential detrimental matrix effects and to calculate the decision limit CCα and the detection capability CCβ of the fungal biosensor assay, spiked and unspiked samples were analyzed in parallel. All sample measurements were corrected with signals obtained from ethanol and fungal cultures not challenged with estrogen and the reagent blank signal. Spiked samples were prepared at three different days in three technical replications and analyzed with the fungal biosensor assay. According to the EC decision 2002/657 [45], we used the mean of 8 unspiked samples plus 3 times the according standard deviation as base for the decision limit CCα (α = 1%) and used the CCα plus 2 times the standard deviation of the spiked samples as base for the CCβ (β = 5%). As control methods EIA for estrogens and the XenoScreen (Yeast assay) were used with extracted and non-extracted samples.

2.2.4. Specificity

The specificity of the fungal biosensor assay was determined by using the natural estrogens 17β-estradiol, estrone, 17α-estradiol and the conjugated estrogens estrone sulfate and estrone glucuronide. Conjugated substrates were in parallel deconjugated using molluskan β-glucuronidase with sulfatase activity (Sigma Aldrich, Germany). In addition, 10 feed samples were taken directly from the diet of the study animals and dried for 4 h at 104 °C. This treatment did not inactive the estrogenic activity of spiked samples (data not shown). The composition of the ration on dry matter (DM) basis is presented in Table 1. The completely dry feed was ground to a fine powder and tested with the fungal biosensor assay to evaluate the presence of estrogenic activity which may be derived from phytoestrogens of leguminoses present in the diet.

2.2.5. Stability

To determine the stability of sample matrices and spiked samples of

Table 1
Composition of the feed ration on dry matter (DM) basis.

Ingredient	% of diet DM
Corn silage	33.8
Lucerne silage	33.4
Grass silage	22.8
Maize grain	4.0
Protein feed	2.2
Wheat grain	1.6
Hay	1.2
Rye grain	0.5
Minerals	0.3
	100.0

milk, urine, feces, saliva, and plasma, aliquots of 2 mL were kept at −20° and at 4 °C for 6 months. Periodically, matrices and spiked samples were thawed and tested with the fungal biosensor assay.

2.2.6. Enzyme immunoassay

The enzyme immunoassay (EIA) was performed according to Möstl et al. [46] with 50 µL sample extract per reaction. The antibody used in this assay shows a cross reactivity to estrone, 17β-estradiol and 17α-estradiol. All matrices used in the EIA were extracted prior to measurement. Urine samples were prepared as previously described [47]. In brief, 250 µL of urine were added to 300 µL of sodium acetate buffer and hydrolyzed with β-glucuronidase aryl-sulphatase (Sigma, Germany). After incubation over night at 38 °C, 5 µL of diethylether were added followed by centrifugation at 5 °C for 15 min. The ether was dried and the sample was resolved in 300 µL of assay buffer.

Fecal samples were prepared as previously described [46]. In brief, 0.5 g of fecal matter were extracted with 4 mL of 100% methanol and 0.5 mL of distilled water. From this solution 1 mL was dried and 1.2 mL of 1 M KOH and 1 mL of *n*-Hexan/Chloroform (v/v 1:1) was added. After vortexing, 200 µL of acetic acid (90% solution) and 5 mL of diethylether were added and the mix was stored at −20 °C. The organic layer was decanted into a new reaction tube and dried under a nitrogen stream. Then 5 mL of diethylether was added and the extraction was repeated. After the second extraction step, 300 µL of assay buffer were used to resolve the sample.

Blood plasma (1 mL) was extracted using 5 mL of diethylether. After storage at −20 °C, the organic phase was transferred into a new tube and evaporated under a nitrogen stream. The extraction was repeated two times, and finally the ether was dried and 300 µL of assay buffer were added to resolve the sample.

Saliva was extracted as described for blood plasma, with the variation, that 900 µL of saliva were used.

Milk was extracted by adding 5 mL of diethylether to 1 mL of milk. After storage at −20 °C, the organic phase was transferred into a new tube and evaporated under a nitrogen stream. After a second extraction step, 1.5 mL of 1 M KOH and 1 mL of *n*-hexane-chlorophorm (v/v 1:1) was added. After vortexing, 200 µL of acetic acid (90% solution) and 5 mL of diethylether were added and frozen. The organic layer was decanted into a new reaction tube and dried under a nitrogen stream. Then, 5 mL of diethylether were added and the extraction was repeated. After the last extraction step, 300 µL of assay buffer were used to resolve the sample.

2.2.7. XenoScreen XL YES assay

The XenoScreen XL YES for the detection of estrogenic activity by yeast cells was obtained from Xenometrix (Switzerland) and performed according to the manufacturer's protocol.

2.3. Assay validation

Prior to screening of clinical bovine samples the test system was validated with spiked samples following the EC Decision 2002/657 for estrogenic active banned substances by determining the detection capability (CCβ), specificity and stability in all matrices [48]. Furthermore the test was established in a high-throughput format to fulfil the requirements for high-throughput prescreening (HTPS) set by the EDSTAC report (1998) for estrogenic activity measurements. The fungal biosensor assay's sensitivity and specificity was calculated using a 2 × 2 contingency table and the sensitivity (SENS) as test system was calculated with: SENS = 100 * number of true positives/(number of true positives + number of false negatives) and the specificity (SPEC) of the test system was calculated with: SPEC = 100 * number of true negatives/(number of false positives + number of true negatives).

2.3.1. High pressure liquid chromatography (HPLC)

The extracts were resolved in acetonitrile and cleaned on a Sep-Pak C18 column (waters, USA). The eluate was analyzed on a HPLC-DAD system (LC-20AD, Shimadzu, Austria) coupled with a DAD (SPD M 20A) using a Nova-Pak, 5µm C18 (Waters, USA) column. Separation was performed at 20 °C using a column oven (CTO20A, Shimadzu, Austria) with a linear gradient starting at 20% rising to 40% of Acetonitrile in phosphate buffer (0.01 M, at pH 7). Absorbance of fractions was measured at 280 nm.

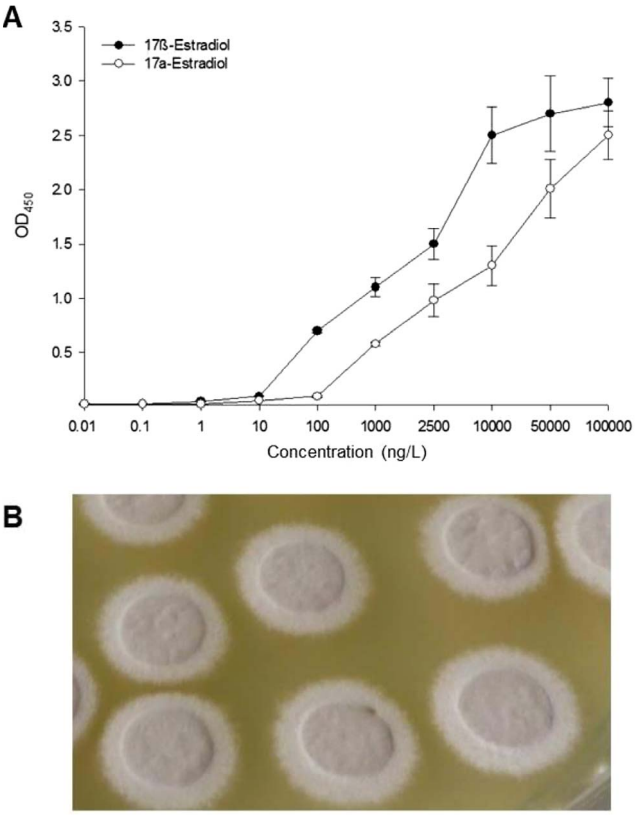

Fig. 1. Response of the *Aspergillus nidulans* biosensor strain to 17β-estradiol and 17α-estradiol after 6 h of exposure (A). The fungal biosensor was exposed to 3 µL of 17β-estradiol in ethanol which was pipetted directly onto the fungal culture spots (B). Signals are the mean of three technical replications with the respective standard deviation and have been corrected with the recorded blank and ethanol values.

3. Results

3.1. Assay characteristics

The sensitivity and reproducibility of the fungal biosensor assay for ethanol solved standards were assessed by using dilutions of 17β-estradiol, the main active estrogen in the reproductive cycle, in 96% ethanol (0.01 ng/L to 100 mg/L; Fig. 1A). The fungal culture spots of the highly-sensitive ERE-URA-RS reporter strain were directly challenged with 5 µl of the dissolved compounds (Fig. 1B). Every measurement was performed in three technical and three biological replications on three different days. Using the qualitative approach with the X-Gal staining, the limit of detection was influenced by the time the color reaction needed to develop and the intensity of the visually observed color. The limit of detection was 850 ng/L of 17β-estradiol or – correlated to the applied volume – an absolute amount of approximately 2.5 pg was detected by the biosensor. Lower concentrations were not discriminable visually alone from the developing background signal in the highly sensitive strain used. Using the quantitative method, the limit of detection was calculated to be 1 ng/L or an absolute amount of 5 fg of 17β-estradiol, and the response of the fungal biosensor assay to the concentrations range was highly reproducible.

The specificity of the biosensor was determined by the ability to

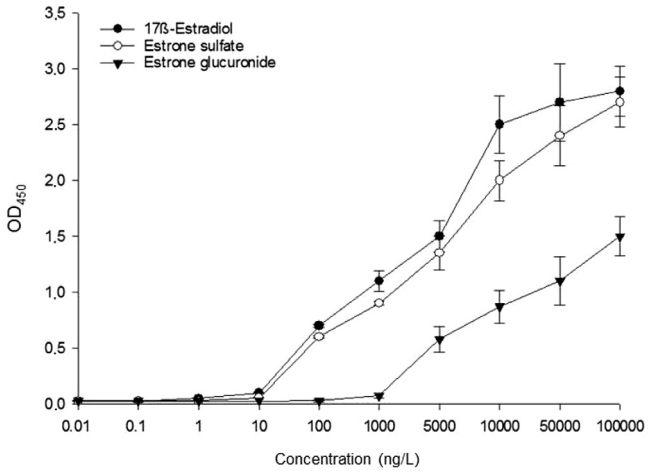

Fig. 2. Response of the *Aspergillus nidulans* biosensor strain to estrone sulfate and estrone glucuronide after 6 h of exposure. Signals are the mean of three technical replications with the respective standard deviation and have been corrected with the recorded blank and ethanol values.

Table 2
Limit of detection of the biosensor assay for tested estrogenic hormones.

Tested estrogenic hormone	Limit of detection (ng/L)
17β-Estradiol	1.0
Estrone	2.5
17α-Estradiol	10.0
Estrone sulfate	10.0
Estrone glucuronide	100.0
Deconjugated estrone sulfate	9.0
Deconjugated estrone glucuronide	95.0

respond to estrone, 17α-estradiol (Fig.1A), and to the conjugated estrogens, estrone sulfate and estrone glucuronide (Fig. 2). The limit of detection for estrone was comparable to that for 17β-estradiol. The limit of detection for 17α-estradiol was observed at 10 ng/L, which indicates less specificity of the receptor. The limits for the conjugated estradiols were observed a concentration of 10 ng/L for estrone sulfate and 100 ng/L for estrone glucuronide. The conjugated estrogens were further deconjugated with glucuronidases and sulfatases from limpets which lead only to slightly increased signals in the fungal biosensor assay (Table 2).

3.2. Assay validation

The fungal biosensor assay was validated for the ability to detect estrogenic activity in non-extracted milk, urine, feces, blood and saliva. Samples of the respective matrices were used from the same time points. Three milk samples were chosen from 6 cows, dividing the samples into pre-, main- and post-milk and the estrogenic activity was evaluated. The calculated mean value using 17β-estradiol standards of 6 samples of main-milk was 8 ± 2.4 ng/L of estrogen. The estrogen level measured by the control EIA assay displayed for the same 6 milk samples after chemical extraction a mean value of 9.71 ± 3.49 ng/L of estrogen. The pre-milk samples displayed no significant difference to the main-milk samples. However, the reproducibility of the post milk samples varied compared to the main-milk sample results (data not shown). Thus, for further validation main-milk was chosen. The fungal biosensor assay recorded a mean base estrogen activity level of 620.4 ± 90.9 ng/L for the fecal samples and the EIA recorded a mean base level of 580 ± 140.2 ng/L of estrogen. The fungal biosensor recorded 375.5 ± 98.4 ng/L and the EIA recorded 348.5 ± 110 ng/L in urine samples. Saliva and plasma samples contained insufficient levels of estrogenic activity to be detected by either the fungal biosensor assay

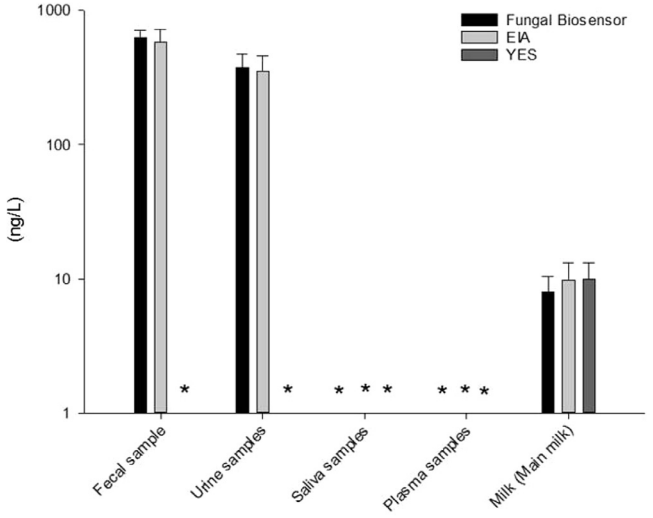

Fig. 3. Comparison of the response of the *Aspergillus nidulans* biosensor strain, the YES assay to unextracted spiked sample matrices and the reference method EIA with extracted sample matrices to 17β-estradiol. Signals are the mean of three technical replications with the respective standard deviation and have been corrected with the recorded blank and ethanol values. Asterisks indicate non-detectable values.

or the EIA (Fig. 3). The estrogen concentrations in non-extracted milk obtained by the Xenoscreen assay were comparable to those obtained by the fungal biosensor strain. However, non-extracted urine and feces led to no results using the Xenoscreen assay (Fig. 3). To determine the presence of natural occurring estrogens in the bovine feces, an HPLC separation method, which was able to separate 17β-estradiol from 17α-estradiol (estron fraction 55–57; estradiol 17α fraction 47–49; estradiol 17β fraction 43–45), was established and 90 collected fractions containing increasing amount of acetonitrile were screened with the fungal biosensor assay (Fig. 4A). The fungal biosensor assay recorded estrogen activity respectively in the fractions containing the estrogens, which was confirmed with the EIA assay and the retention time of estrogen standards. A feed sample was resolved in ethanol and tested for estrogenic activity with the fungal biosensor assay. The sample displayed a strong estrogenic activity of 640 ± 84 ng/L which indicated the presence of phytoestrogens or other estrogenic active compounds. The dried feed sample was further extracted and separated using the HPLC protocol established. Testing of the feed fractions with the fungal biosensor assay revealed several estrogenic active fractions (Fig. 4B). The estrogenic active fractions observed in feces eluted at higher organic phase content than the estrogenic active fractions observed in feed using the same separation protocol. Weak signals at the retention time observed in feed occurred in feces as well.

3.3. Performance characteristics

To validate the fungal biosensor strain as bioassay, the decision limit CCα, the detection capability CCβ and the stability of the assay in the respective matrices were evaluated according to the EC decision 2002/657 for banned estrogenic active substances. Milk, urine, and fecal samples were spiked at a level of 15 and 1.5 ng/L with 17β-estradiol and CCα and CCβ were calculated for all three matrices (Tables 3 and 4). Spiking with the lowest concentration of 1.5 ng/L led to significant signals in all three tested matrices. The stability of the spiked samples was evaluated every three weeks for a period of 6 months (8 time points). Milk was kept at 4 °C. Feces and urine samples were frozen and kept at −20 °C until further use. After fouling started in the milk samples, usually after ten days, the results of the fungal biosensor assay became inconclusive (data not shown). Freezing of the milk samples lead to no detectable amount of estrogen after mixing with ethanol. The lipid phase remained separated, which indicated that the lipophilic

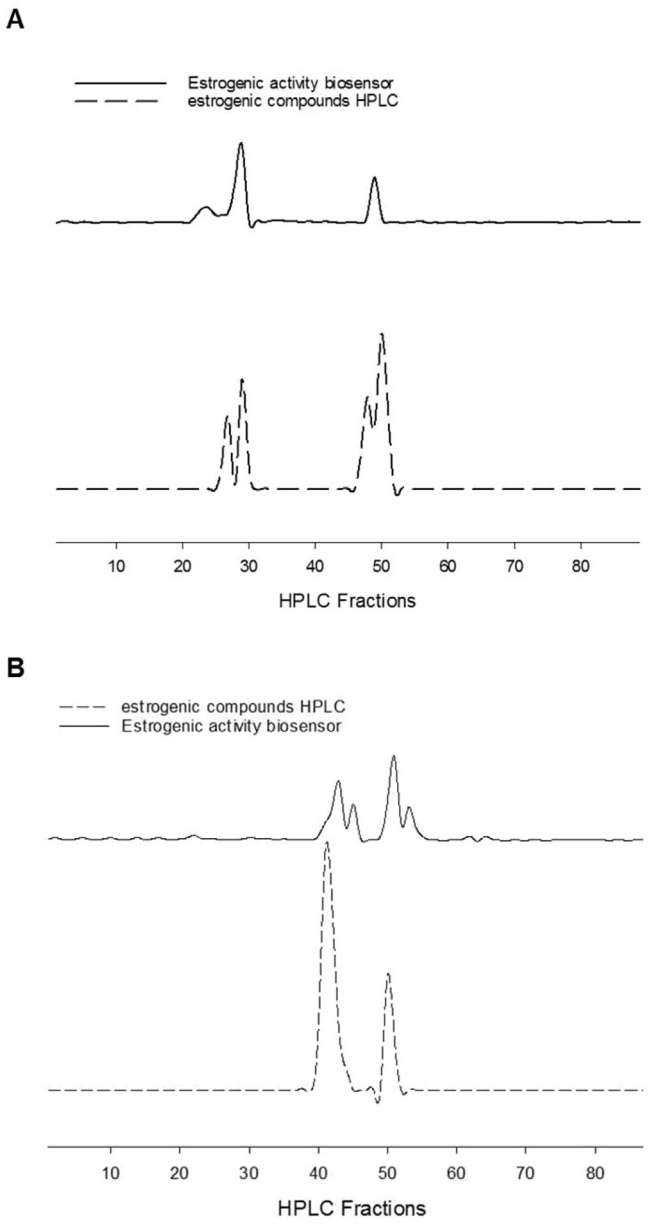

Fig. 4. HPLC fractions of bovine feces (A) tested with the *Aspergillus nidulans* biosensor strain for the presence of estrogenic active fractions. HPLC fractions of feed from the test animal diet (B) tested with the *Aspergillus nidulans* biosensor assay for the presence of estrogenic active fractions.

estrogen compounds remained in the insoluble lipid phase. Feces and urine displayed no decline of estrogenic activity after freezing and during storing.

3.4. Clinical applicability of high through put evaluation of pregnancy

The fungal biosensor assay was used to determine pregnancy of mares and cows due to the estrogen activity in the respective fecal samples. Fecal samples of 140 randomized mares were used to predict pregnancy. Extracted fecal samples were tested with the EIA, defined as gold standard. Non-extracted fecal samples were mixed with ethanol (1:1 w/v) and the supernatant was directly used for the fungal biosensor assay. According to the EIA assay, 18 mares were evaluated as pregnant. Using the fungal bioassay, pregnant mare samples showed in all but one case a signal at least 3 times higher than the observed background signal of the fungus. Thus, we chose a cut-off value for estrogenic activity above 0.1 at 420 nm. Using this cut-off the fungal biosensor assay predicted 17 pregnant individuals and 1 false negative individual (sensitivity: 97.4%, specificity 100%). The fungal biosensor assay was further evaluated to predict pregnancy in cow fecal samples. Thus, 149 fecal cow samples were tested with the fungal biosensor for elevated estrogenic activity. As gold standard for pregnancy diagnosis, cows were examined ultrasonographically at day 39 after artificial insemination (AI) and a second pregnancy diagnosis was performed at day 92 after AI. The biosensor predicted 129 pregnant cows and 2 false negative cows (sensitivity: 99.2%, specificity 100%). Pregnancy was detectable from the first quarter to the third quarter. However stronger overall signals were received starting from the second quarter to the third. The false negative results were derived from the third and the second quarter of pregnancy. This indicates a technical issue in sample preparation.

The high-throughput adaption of the fungal biosensor assay was realized on a robotic pipetting unit (Hamilton, StarLet). Up to five 96 deep well plates can be processed per run, which takes approximately 2 h including the preparation before and after the automated sequence. Thus, up to 1440 samples can be processed per day with these units.

4. Discussion

Estrogenic compounds have been identified in a wide range of environmental sources and consumer products. Drainage water from cattle pasture, for example, contains up to 14 ng/L 17β-estradiol equivalents measured by the yeast estrogen screen (YES) [49]. In commercial whole milk from cows, 13.6 pg/mL estrone and 2.7 pg/mL 17β-estradiol were detected by High performance liquid chromatography-tandem mass spectrometry [50]. With a detection limit of 1 ng/L for 17β-estradiol and estrone, the fungal biosensor assay is a promising tool for a sensitive screening of a wide range of matrices for the presence of estrogenic active compounds. The ability of the chromosomally integrated *hERα* receptor to react to various naturally occurring estrogens, i.e. estrone, estradiol, estriol and the conjugates estrone sulfate and estrone glucuronide, enables the determination of the total estrogenic activity in the investigated matrix. In milk, the estrogenic activity derives from natural occurring free estrogens and their respective glucuronated and sulfated metabolites. However, of the tested estrogens, 17β-estradiol has been shown to lead to the highest signal in the fungal biosensor screen which is supported by the findings in yeast based receptor screens [51].

The presented data and determined characteristics of the fungal biosensor assay prove that the assay is able to detect low levels of estrogenic activity (1 ng/L for 17β-estradiol), which is comparable to yeast based screening systems (2 ng/L) [31]. Furthermore, the assay is able to detect an increase of 1.5 ng/L in non-extracted feces, milk and urine originating from cows. The signal of all spiked samples in the respective matrices was higher than the calculated CCα value and the mean response was higher than the calculated CCβ value for the respective matrix. Thus, there is less than 5% of probability of a false decision when the matrices contain equal or higher amounts of estrogen activity.

The fungal biosensor displayed a weak sensitivity (100 ng/L) for estrone glucuronide and moderate sensitivity (10 ng/L) for estrone sulfate, indicating that the sulfate residue does not influence the receptor binding as strong as the glucuronide residue. It was shown in *Aspergillus niger* that highly specific glucuronidases are secreted [52]. However in this study no indications for conversion of estrogens have been observed. In *Aspergillus nidulans* the presence of glucuronidase genes originating from bacteria has been confirmed in silico [53]. Deconjugation by glucuronidase and sulfatase of milk, urine and feces did only marginally increase the detected estrogen activity of the samples. This can be explained by the fact that in feces mainly unconjugated estrogens are present [54] and that in urine the major estrogen metabolite is estrone sulfate [55], for which the fungal test system showed moderate sensitivity. Milk mainly contains conjugated estrogen

Table 3
Mean estrogenic activity triggered fungal biosensor assay signal of 8 blank samples derived from cows and the determination of the decision limit CCα.

Sample type	Mean signal OD_{420}	SD	CCα	Sample number							
				1	2	3	4	5	6	7	8
Milk											
Blank	0.072	0.005	0.084	0.068	0.078	0.069	0.075	0.076	0.075	0.064	0.074
E2 1.5 ng/L	0.096	0.006		0.098	0.101	0.089	0.092	0.095	0.100	0.089	0.104
E2 15 ng/L	0.118	0.005		0.118	0.120	0.125	0.124	0.115	0.110	0.120	0.114
Feces											
Blank	0.840	0.061	1.020	0.790	0.801	0.890	0.750	0.840	0.810	0.768	0.740
E2 1.5 ng/L	0.925	0.021		0.910	0.940	0.960	0.910	0.945	0.914	0.901	0.920
E2 15 ng/L	1.028	0.032		1.020	1.040	1.070	1.020	0.990	1.040	1.060	0.980
Urine											
Blank	0.500	0.019	0.557	0.540	0.490	0.510	0.480	0.520	0.510	0.490	0.520
E2 1.5 ng/L	0.586	0.018		0.610	0.570	0.590	0.560	0.600	0.590	0.570	0.600
E2 15 ng/L	0.678	0.020		0.710	0.660	0.680	0.650	0.690	0.680	0.660	0.690

SD, standard deviation; CCα, decision limit

metabolites [56,57] and therefore, it remains unclear why in the present study deconjugation did not lead to an increased estrogenic activity. We speculate that the naturally occurring bacterial flora rapidly degrades the conjugated estrogens.

In this study, we could not detect estrogenic activity in saliva or plasma. The amount of estrogen in both matrices is probably below the limit of detection of 1 ng/L of the biosensor assay and the used control assays (EIA and YES assay). Monk et al. [36] recorded on average three-times higher estrogen (estrone and estradiol) levels in bovine milk (80 pg/mL) compared with plasma (26 pg/mL) throughout the estrous cycle using RIA. A strong positive correlation was observed between estrogen concentrations in saliva, blood plasma and milk with highest concentrations in milk. The estrogen concentrations in serum ranged from 7 to 31 pg/mL, saliva from 10 to 35 pg/mL and milk from 10 to 43 pg/mL throughout the estrous cycle [58]. The discrepancies between studies may be because of different methods used for the estrogen measurement. The bovine fecal and urine samples used for validation of the fungal biosensor assay showed higher amounts of estrogen activity compared to the milk samples. Studies using radiotracers to investigate estrogen excretion have shown that the main excretion route in cows is feces (58%) and urine (42%) [38]. In addition, it has also been shown that phytoestrogens in the diet of the animals can effect fecal estrogenic activity [59]. To avoid the potential detection of phytoestrogens from the diet of the study animals, we screened HPLC fractions of feed and fecal samples for estrogenic activity. Using the fungal biosensor, different fractions of estrogenic activity were detected in feces compared with the feed samples. Signals at the retention time of standard estrogens were observed in feed and feces as well. This indicates that some phytoestrogens would influence the biosensor. However the second strong signals from feed do not appear in the feces fraction. This observation can be supported by the findings that isoflavones and plant lignans are heavily degraded in the bovine metabolism and only small portions are excreted [60].

As clinical application, the fungal biosensor assay is able to predict early pregnancy 40–60 days of gestation due to the elevated estrogens level in bovine and mare feces. From 140 fecal samples originating from mares, the fungal biosensor assay showed 1 false negative result, which is below the accepted false negative rate of 5%. In case of 149 cow fecal samples, 2 false negative results were observed, which is equally below the accepted 5% rate. This observation indicates that the animal specific differences in the estrogen and phytoestrogen metabolism are neglectable for the prediction of pregnancy due to the estrogen activity in feces.

The relative stability of estrogenic activity in stored samples for milk ten days at 4 °C or until fouling occurs and for the frozen samples at least 6 months shows that the procedure is robust and reproducible. Thus, the fungal biosensor assay meets all the required criteria demanded by the EC Decision 2002/657 for validation of a qualitative screening method [48].

The robustness of the fungal biosensor makes a chemical extraction and clean-up procedure obsolete, which makes it possible to perform the test without organic solvents and toxic compounds. Lysis of the fungal biosensor cells is based on mechanical lysis technology and avoids the use of chemicals. The method is in accordance with the green chemistry principle by preventing solvent waste of clean-up processes and reduction of harmful substances in the usage [61]. The fungal biosensor assay is further faster than comparable yeast based systems which take 3–4 days until a read-out is available.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Table 4
Determination of the detection capability CCβ and the content of estrogenic activity.

Sample type	CCα	Mean signal OD_{420} (X)	SD	CCβ	CCβ criterion met (X > CCβ)	CCβ (ng/L)	Content (ng/L)
Milk							
E2 1.5 ng/L	0.048	0.096	0.006	0.096	Yes	11.080	17.650
E2 15 ng/L	0.048	0.118	0.005	0.095	Yes	11.070	25.490
Feces							
E2 1.5 ng/L	1.020	1.159	0.062	1.143	Yes	780.460	790.850
E2 15 ng/L	1.020	1.509	0.062	1.143	Yes	780.460	946.300
Urine							
E2 1.5 ng/L	0.557	0.597	0.018	0.592	Yes	423.120	429.650
E2 15 ng/L	0.557	0.678	0.020	0.597	Yes	423.120	520.670

SD, standard deviation; CCα, decision limit; CCβ, detection capability.

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