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Validity Test for a Yeast Two-Hybrid Assay to Screen for Estrogenic Activity, and Its Application to Insecticides and Disinfectants for Veterinary Use

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Recently, toxic compounds known as endocrine disrupters (EDs), which cannot be categorized by conventional toxicology, have been identified (International workshop on endocrine disruptors 1997). These substances cause endocrine disruption by acting as agonists and/or antagonists of naturally occurring endogenous physiologically active substances. In practice, the most common EDs are substances having estrogenic activity. Since the 1930s (Astwood 1938), estrogenic substances have been routinely detected in vivo assays using experimental animals (reviewed by Reel et al. 1996). However, these methods are time consuming and expensive. Consequently, several in vitro assays have been developed to determine whether a chemical has estrogenic activity (Soto et al. 1991,1994,1995; Routledge et al. 1996). For example, Nishikawa et al. (1999) developed the yeast two-hybrid assay (YTA), which is based on the ligand-dependent interaction between a nuclear hormone receptor and a coactivator. This method is not only simple and has high sensitivity, but also has good reproducibility and the expression mechanism is close to a human one. However, damage of the yeast by the test substance during the assay may result in a false negative. Thus, we designed a "validity test" (VT) to validate the two-hybrid assay by including a positive control in which a known estrogenic compound was mixed with the test substance. This modification was combined with an alternative statistical analysis to determine whether the test substance had caused a reduction in the viability of the yeast, leading to a false negative. Using this method, we tested the estrogenecity of active ingredients of veterinary medicinal products of several pyrethroid insecticides, disinfectants and anthermintics.

MATERIALS AND METHODS

The yeast transformant was produced following Nishikawa's method (Nishikawa et al. 1999). The yeast expression plasmids, pGBT9 and pGAD424 were purchased from Clonetech (Palo Alto, CA). The ER α and TIF2 were used in the

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assay as estrogen receptor and its coactivator. *Saccharomyces cerevisiae* Y190 (*MATa, ura3-52, his3-D200, ade2-101, trol-901, leu2-3, 112, gal4Dgal80D, URA3:::GAL-lacZ, cyhr2, LYS2:::GAL-HIS3*) was obtained from Clonetech (Palo Alto, CA) and transformed with the pGBT9-rER α and pGAD424-TIF2 using a lithium acetate method. All reagents were reagent grade or better and were purchased from Difco Laboratories (Detroit MI), Seikagaku Co., Ltd. (Tokyo Japan), Wako Pure Chemical Industries Ltd. (Osaka Japan), Sigma Chemical Co. (St. Louis MO) and Kanto Chemicals Co., Ltd. (Tokyo Japan). Test substances were kindly donated by the following companies: permethrin (PER, 96.0% pure), Osaka Seiyaku Co., Ltd. (Osaka Japan); pyrethrins (PYR, 20.19%), Gendai Seiyaku Corp. (Tokyo Japan); resmethrin (RES, 95.1%), Yashima Sangyo Co., Ltd. (Kanagawa Japan); phenothrin (PHE, 96.1 %), Osaka Seiyaku Co., Ltd. (Osaka Japan); phthalthrin (PHT, 94.4%), Yashima Sangyo Co., Ltd. (Kanagawa Japan); polyoxyethylene alkylphenylether (POE, 53.7%), Younichi Chemical Institute Co. (Nagoya Japan); benzalkonium chloride (BC, 96.4%), Koyo Chemical Industries, Ltd. (Hyogo Japan); polyalkyl polyaminoethyl-glycyne hydrochloride (PAP, 53.0%), Yuko Chemical Industries Co., Ltd. (Hyogo Japan); didecyldimethylammonium chloride (DMA, 73.8%), Scientific Feed Laboratory Co., Ltd. (Tokyo Japan); quinomethionate (QM, 91.7%), Scientific Feed Laboratory Co., Ltd. (Tokyo Japan); chlorhexidine digluconate (GC, 20%), purchased from Wako Pure Chemical Industries Ltd.(Osaka Japan); o-dichlorobenzene (ODB, 100%), Scientific Feed Laboratory Co., Ltd. (Tokyo Japan); nonoxynol iodine (NI, 1.75%), Koyo Chemical Industries, Ltd. (Hyogo Japan); glutaraldehyde (GA, 51.5%), Yashima Sangyo Co., Ltd. (Kanagawa Japan); ivermectin (IVE), purchased from Sigma Chemical Co.(St. Louis MO). YTA was performed following Nishikawa's method (Nishikawa et al., 1999) with minor modifications. In brief, the yeast was pre-cultured in 5mL of liquid SD medium (lacking tryptophan and leucine) overnight at 30°C with shaking (100 rpm) in a L shape glass tube (a 20mm i.d. and 130mm long horizontal tube). Test substance were dissolved in 10 μL of DMSO, added to 190 μL of liquid SD medium (lacking tryptophan and leucine) and mixed with 50 μL of pre-cultured yeast in a 1.5mL centrifuge tube, and incubated for 4 hr at 30°C. After measuring absorbance at 595nm, the cell were washed by centrifugation and lysed. 96 well microplate and microplate reader was used to measure all of absorbance. To lyse the yeast, 200 μL of Zymolyase 20T (1mg/mL) dissolved in Z buffer (0.1M sodium phosphate [pH=7.0], 10mM KCl and 1mM $MgSO_4$) was added to centrifuged cell and left it standing for 15 min at 37°C. The lysate was added 4mg/mL ONPG (40 μL) and shaked at 30°C until coloring. After quenching the reaction by 1M Na_2CO_3 (100 μL), it was centrifuged and its supernatant was

measured the absorbance at 570nm and 415nm. β-galactosidase activity (U) was calculated from the absorbance. Estrogenic acitivity of the test substance was expressed as the concentration required to give an activity equivalent to 10% of the activity of 0.01mg/L (3.67×10^{-8}M) 17β-estradiol (E2) (REC10m). The VT procedure was the same as the YTA procedure, except 0.01mg/L E2 was mixed with the test substance. If the test substance did not damage the yeast and had no estrogenic activity, U in the YTA procedure would be equivalent to that obtained in controls in which 0.01mg/L E2 alone was added to the YTA. Greater toxicity of the test substance leads to a reduced response in the VT. If the U in the VT was less than 97.5% (one side) confidence limits of the negative controls (T), the corresponding YTA of the test substance was deemed invalid. That is

$$E = 0.1 \times P \times \frac{(I - Bi) - (R - Br)}{P - Bp} \qquad (1)$$

P : U at 0.01mg/L of E2
I : U of test sample in the VT
R : U of test sample in the YTA
Bp : Background for P
Bi : Background for I
Br : Background for R

(1) was intended to exhibit a rate to suppress the U by the test substance, and E means a supposed U suppressed by the test substance itself if the concentration of tested substance were to REC10m. When P,I and R get at the same experiment,

$$Bp = Bi = Br = B \qquad (2)$$

and (1) is to

$$E = 0.1 \times P \times \frac{I - R}{P - B} \qquad (3)$$

If E is higher than the background level significantly, the run is determined realizable. And we determined

$E > T$: Corresponding YTA is valid.
$E \leqq T$: Corresponding YTA is invalid.

T is the confidence limit (α=0.025) for the mean of the negative controls in the VT assuming a normal distribution. In this case (n=42), T was 18.7.

Table 1. Results of yeast two-hybrid assay and validity test for active ingredients of disinfectants and insecticides for veterinary use in Japan.

Category	Tested Substance	REC10m (mg/L)*
pyrethroids	permethrin (PER)	>100
	pyrethrins (PYR)	>100
	resmethrin (RES)	>100
	phenothrin (PHE)	>100
	phthalthrin (PHT)	>100
	allethrins (ALE)	>100
disinfectants	polyoxyethylene alkylphenylether (POE)	>10
	benzalkonium chloride (BC)	>1
	polyalkyl polyaminoethylglycyne hydrochloride (PAP)	>10
	didecyldimethylanmonium chloride (DMA)	>1
	quinomethionate (QM)	>0.1
	chlorhexidine digluconate (GC)	>10
	o-dichlorlobenzene (ODB)	>100
	nonoxynol iodine (NI)	>10
	glutaraldehyde (GA)	>1
other	ivermectin (IVE)	>100

*REC10m is the concentration of test substance eliciting a response equivalent to 10% of the response elicited by 0.01mg/L E2.

RESULTS AND DISCUSSION

The substances tested in this study are all approved in Japan as active ingredients of veterinary medicinal products. IVE was also tested as a precaution because Lacau-Mingido et al. (2000) reported continuous IVE treatment in grazing dairy heifers may affect endocrine secretions. A range of concentrations (0.01, 0.1, 1, 10, 100mg/L) of each test substance was analyzed in duplicate and each test was performed three times in total. No substance showed a response greater than 10% of that shown by 0.01mg/L of E2 in the YTA. In addition, no deleterious effect was observed in the VT by any concentration of the pyrethroids, or ODB and IVE. Thus, we demonstrated that the YTA is possible with up to 100mg/L of these substances (Table 1.). On the other hand, POE, PAP, GC and NI were deleterious to the assay at concentrations of above 10mg/L, and BC, DMA, and GA at concentrations of above 1mg/L. The most toxic substance was QM, which

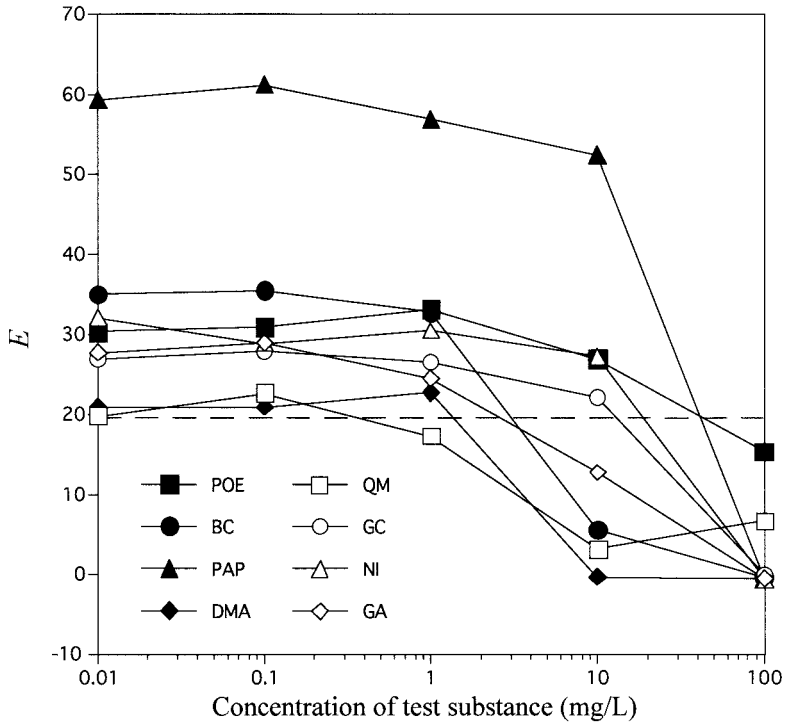

Figure 1. Relationship between *E* and the concentration of each test substance. The dotted line is the top limit of the confidential interval for the negative controls. The substances that did not show any effects are omitted.

was found to be deleterious in the VT at concentrations above 0.1 mg/L. As expected, the *E* values of the deleterious test substances decreased with increasing concentration (Figure 1.). These results agree with those of Nishihara et al. (2000) who reported that several of these substances do not demonstrate estrogenic activity in the YTA. However, we have shown by the VT that the maximum concentration of the disinfectants that can be tested in this assay is lower than that tested by them. Other investigators have reported that some of the pyrethroids tested in this study have estrogenic activity in different tests. For example, Go et al. (1999) reported that phenothrin (sumithrin), allethrin, permethrin have estrogenic activity at high concentrations in a test using MCF-7 cells. Estrogenic activity has also been observed for phenothrim at about 10mg/L in a test using a human endometrial cancer cell line (Garey et al. 1998). However, we did not detect estrogenic activity in these compounds using the YTA, and none of them proved to be toxic in the VT assay at the concentrations tested. Therefore for the pyrethroids, the sensitivity of the YTA may differ from that of other methods.The disinfectants showed some toxicity for the yeast cells in the VT, but the

pyrethroids did not. Thus, we can say none of the disinfectants displayed estrogenic activity at concentrations that can be tested without damaging the yeast cells. Concentrations above this range are similar to those used for disinfection. Thus, further toxicity studies of the disinfectants should consider possible synergy of conventional toxicity and estrogenic activity. Cell growth in the presence of a test substance is often used to estimate toxicity for live cells. However, the cell growth test will not determine whether the YTA is valid when the test substance is slightly toxic. Using the VT we can determine whether the YTA is valid or not using a statistical analysis. Nevertheless, test substances that have antagonist or partial agonist activity may compromise the VT. This limitation can be overcome by measuring the absorbance at 595nm of the preculture liquid and test substance after incubation, which will reveal whether the yeast cells have been damaged or not. Using this method, it may also be possible that the VT test could predict agonist activity of the test compound if the yeast cells are not damaged. However, no substances were suspected of having antagonist activity in this study.

Some reports have described estrogenic activity of disinfectants. Nonoxynol, one of the main components of NI, is thought not to have estrogenic activity, but is metabolized in animals to form nonylphenol, which has been identified as an ED (Sonnenschein et al. 1998). Other disinfectants have biodegradable structures similar to surfactants. Thus, the degradation products from these compounds should also be examined for estrogenic activity when considering their toxicity. If synergistic toxicity between the test substance and E2 occurs, the valid maximum concentration of the test substance for the YTA will be underestimated by the VT. However, synergy between the test substance and E2 will not cause an error in the YTA for valid concentrations of the test substance. Failure of the test substance to be taken up by the yeast cells would result in a large error in the YTA. However, this is not an issue for compounds that display toxicity in the VT. These factors should be considered when apply the YTA and VT tests to determine the estrogenic activity of specific test substances. In general, all assays for estrogenic activity have specific advantages and disadvantages that should be considered before applying these tests.

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