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CHARACTERIZATION OF RICE FALSE SMUT PATHOGEN *USTILAGINOIDEA VIRENS* AND ITS USTILOXIN TOXIN IMPACT ON BRINE SHRIMP

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ABSTRACT

Survey on rice false smut disease has been conducted at Erode, Tanjore, Perambalur and Coimbatore districts of Tamil Nadu, India. Among the areas surveyed, higher disease incidence was recorded in the paddy variety bhavani cultivated in Bhavanisagar, Erode district in which the rice false smut disease incidence was recorded as 40%. The pathogenicity test revealed the symptom development within 12-15 days of conidial suspension injection. In order to characterize the fungal isolates of *Ustilaginoidea virens*, a causal for rice false smut disease was cultured on Potato Sucrose Agar (PSA) medium. The pathogen was characterized morphologically which exhibits white mycelium that turns yellow and becomes olive green on maturity. Molecular confirmation was carried out among the collected isolates that were amplified at 380 bp region which is found specific for the pathogen *Ustilaginoidea virens* using the primers US 1-5 and US 3-3. The extraction and purification of ustiloxin was carried out using flash chromatography. On purification 100% water and 30% ethanol fraction was collected separately. The cytotoxicity assay of ustiloxin in brine shrimp resulted that the mortality at 1% concentration of ustiloxin was 96% and the lowest mortality (26%) was observed at 0.2% concentration. The lethal dosage 50(LD_{50}) was confirmed at the concentration of 0.4% ustiloxin having the mortality rate of 52% using brine shrimp bioassay.

KEY WORDS : False smut, Disease incidence, Characterization, Extraction, Purification, Cytotoxicity.

INTRODUCTION

Rice is one of the most important food crop that provides staple food for more than half of the global population. In recent years, the production and productivity of rice is decreased due to environmental concerns such as water scarcity, climate change, global warming, pollution, biotic and abiotic stress. Among the various biotic stresses, the most important sink infecting ascomycetes fungal disease is rice false smut caused by *Ustilaginoidea virens,* Cooke (Takahashi, 1896) and its teleomorphic stage is *Villosiclava virens* (Tanaka and Tanaka, 2008) leads to devastating yield loss. First report of rice false smut (RFS) disease from Tirunelveli district of Tamil Nadu (Cooke, 1878). Rice false smut causes high yield loss due to planting of high fertilizer responsive varieties and hybrids, application of huge nitrogenous fertilizers and more inevitably global climatic change (Ladhalakshmi *et al*., 2012). The fungus produces symptom only in which it transforms the individual grains into yellowish smut balls further to yellowish orange, green, olive green and finally to greenish black (Biswas, 2001). Furthermore, this fungus release the secondary toxic metabolites such as ustiloxins, ustilaginoidins and sorbicillinoids makes it the most harmful disease in recent years (Sun *et al*., 2020; Qiu *et al.,* 2020; Wang *et al*., 2016). Among the three toxic metabolites of the fungus, ustiloxin is the most harmful toxin due to its antimitotic properties in eukaryotic cells and found to have a variety of negative consequences on plants, animals and humans (Koiso *et al*., 1994). Ustiloxin mostly exist in

powdery chlamydospores, which are easily released into the environment by air currents and rain splashes, creating new environmental challenges due to its hydrophilic nature (Fan *et al*., 2016). More than 40 rice growing countries have reported the outbreak of RFS worldwide (Ladhalakshmi *et al*., 2012) implicating the exposure of ustiloxin in both the environment as well as among human beings. Additionally, ustiloxin levels detected in serum and urine samples taken from the inhabitants of Xianfeng (Enshi City, Hubei Province, China) (Sun *et al*., 2022). The purification of ustiloxin from rice false smut balls can be carried out using the macroporous resins SP207 and SP700 (Shan *et al*., 2013), polymer cation exchange resins and polymer anion exchange resins (Cao *et al*., 2016), Following purification, the ustiloxins can be detected through HPLC (High Performance Liquid Chromatography) (Wang *et al*., 2016, Shan *et al*., 2013, Sun *et al*., 2022, Hu *et al*., 2020), LC-MS (Liquid chromatography- Mass Spectrometry) (Sun *et al*., 2021, Sun *et al*., 2022, Shan *et al*., 2012, Cao *et al*., 2016) and ELISA (Enzyme Linked Immuno Sorbent Assay) (Fu *et al*., 2015). Cao *et al*., (2016) and Fu *et al*., (2015) quantified the ustiloxin in peeled rice samples, Cheng *et al*., (2019) quantified the presence of ustiloxin in surface water of rice and Sun *et al*. (2022) quantified the ustiloxin in serum and urine samples of mice and humans using HPLC and LC-MS/MS analysis. Based on the above facts, current article was primarily focused on characterization of the pathogen *Ustilagonoidea virens* and its ustiloxin toxin impact in surface water ecosystem of rice through indirect testing by brine shrimp toxicity assay.

MATERIALS AND METHODS

Survey and collection

Roving survey has been conducted during first quarter of 2023 at some selected rice fields of Tanjore, Erode, Perambalur and Coimbatore districts of Tamil Nadu to calculate Percent Disease Incidence (PDI) for rice false smut disease. PDI and Disease Severity were calculated by the following formulas.

$$\text{Percent disease incidence} = \frac{\text{Number of tillers infected}}{\text{Total number of tillers}} \times 100$$

Disease severity = Percent tillers infected × Percent smut balls

In order to compare the percent disease incidence and disease severity, the number of infected tillers/ paniclesand the number of smut balls per panicle was recorded. Data are represented in mean. Rice false smut balls were collected for *Ustiloginoidea virens* isolation and toxin extraction. The false smut balls were collected in the sterile 225 gauge transparent zip seal bags and labelled. The samples of false smut balls were immediately kept in icebox and brought to the laboratory. For ustiloxin toxin extraction, false smut balls were dried under shade until to get a constant weight and stored at -20 °C.

Isolation

Ustiloginoidea virens pathogen which has been causal of rice false smut disease was isolated from false smut balls collected from different districts of Tamil Nadu as per the procedure mentioned by Ladhalakshmi *et al*. (2012). Initially, surface sterilization of infected spikelets was carried out using 1% sodium hypochlorite for a minute and washed repeatedly thrice with sterile distilled water. Using a sterilized scalpel, the smutted balls was cut into small pieces and placed at the centre of the plate containing PSA medium (Potato =200g, Sucrose =20g, Agar agar =20g, Distilled water =1000 ml). To avoid bacterial contamination chloramphenicol (0.3g/ 1000 ml) was added to the PSA medium. The plates were incubated for 7-20 days at 27 °C for pathogen growth. After growth of the pathogen, the mycelial disc was taken by using sterilized cork borer and placed on the centre of the Petri plate containing PSA medium to get pure culture of the pathogen.

Characterization

Morphological characterization

Isolates collected from Erode, Tanjore, Perambalur and Coimbatore districts of Tamil Nadu were grown on PSA medium at 27±2 °C for different durations of 10, 20 and 30 days to know about the morphological characters of *U. virens* such as colony colour, colony diameter, mycelial texture and the shape of spores by observing under stereozoom microscope.

Molecular characterization

DNA extraction of the pathogen

The DNA was extracted from 14-days old pathogen culture as per the procedure described by Ladhalakshmi *et al.* (2012). 100 mg of dried mycelial mat was powdered using liquid nitrogen with the sterilized pestle and mortar and 750 µl of CTAB

buffer was added to the powdered mycelium in 1.5ml eppendorf tube and incubated at 65 °C for 45 mins in a water bath. After incubation, the tubes were vortexed for 5 secs and centrifugation was carried out at 13,000 rpm for 15 mins and supernatant was collected. To the supernatant, equal volume of chloroform : isoamyl alcohol (24:1) was added and further centrifuged at 13,000 rpm for 15 mins. Top clear aqueous phase was transferred to a new eppendorf tube and to which equal volume of ice cold isopropanol was added and incubated at -20! for 2 hrs. Then, the tubes were centrifuged at 13,000 rpm for 15 mins to precipitate the DNA pellet. Supernatant was discarded and DNA pellet was washed using 500 µl of 70% ethanol and centrifuged at 10,000 rpm for 10 mins and then air dried. Then DNA sample was dissolved in 50µl of sterile distilled water and stored in the refrigerator. The final DNA concentration was quantified by Nanodrop (Biodrop).

The specific ITS primers of *Ustilagonoidea virens* mentioned by Zhou *et al*., (2003) was used for PCR reaction. The 10 µl PCR mixture constituting 5µl of master mix (Synergy Scientific Services Pvt Ltd, Chennai), 1 µl of US1-5 (CCGGAGGATACAA CCAAAAAAACTCT), 1 µl of US3-3 (GCTCCAAGT GCGAGGATAACT GAAT), 2 µl of DNA template and 1 µl of sterile water. The PCR amplification was carried out with an initial denaturation at 96 °C for 2 mins, followed by 30 cycles of denaturation at 96 °C for 20 secs, annealing at 53 °C for 30 secs, extension at 70 °C for 30 secs and with the final extension for 7 mins at 72 °C. The amplified products were analysed by 1.2% agarose gel electrophoresis at 80V and 200 mA for 1hr along with 100 bp ladder.

Extraction of ustiloxin from false smut balls

The extraction of ustiloxin was carried out as per the procedure mentioned by Shan *et al*., (2012). The dried smut balls stored at -20 °C were used for toxin extraction. 1kg of powdered false smut balls was extracted with milliQ water thrice (2L of water each time) and shaken vigorously (150 rpm) at room temperature. At the third time of extraction, ultrasonication was carried out for 30 mins for the efficient elution of the ustiloxin. The extract was then filtered through Whatman No.4 filter paper fixed in a funnel mounted on a conical flask. Using rotary evaporator (Heidolph, Germany) the filtrate was concentrated at 60 ! till the filtrate volume was reduced upto 600ml.

Then the concentrated water extract of 100 mL is passed through HP20 macroporous resin column (Diaion HP-20 Resin, 13605, Sigma Aldrich, USA) was packed manually using glass column (25 mm × 250 mm, Biorad Medisys Pvt. Ltd, Bengaluru, Karnataka). The HP20 dry resin soaked and mixed with methanol. Later methanol was replaced with water and allowed to settle for 5- 10 mins. Then the resin slurry was carefully poured into the column without forming any air bubbles. It was then backwashed with deionised water in a slow upward flow to remove air bubbles, debris and makes the resin stratified. The glass column containing HP20 resin connected with flash chromatography (Puriflash XS420, interchem) was used for purification. The Puriflash XS420 includes a quaternary pump, a UV detector, fraction collector with solvent trays and bottles. Initially, the mobile phase composed of 100 % water (mobile phase A) for 30 mins followed by 30% ethanol (mobile phase B) for next 30 mins having the total flow rate of 10 ml/min with the injection volume as 5 ml of concentrated crude extract per run and the UV detection was carried out at 220 nm. The ethanol and water fraction was collected separately in test tubes and each fraction was concentrated upto 10 ml in a rotary evaporator at 60 °C (150rpm) and the optical density value of the fractions was detected at 220 nm using UV-Vis spectrophotometer an it was stored in the refrigerator for further use.

Brine shrimp lethality assay

For testing the biological cytotoxicity of ustiloxin, bioassay was carried out using brine shimp (*Artemia salina*) as per the procedure mentioned by Raj *et al*., (2014) with minor modification. The cyst containing eggs of the brine shrimp were obtained from Central Brackish Water Research Institute (CBWRI), Chennai and stored at 4 °C till it is required for usage. The hatching of brine shrimp's eggs/cysts was carried out by using the artificial sea water solution as a hatching medium. Brine shrimp's eggs/cysts were taken out from refrigerated condition and allow it for room temperature (28-30 °C) for 30 minutes. The artificial seawater was prepared by addition of 34 g of sea salt (Sodium chloride) /1000 ml of distilled water and sterilized using autoclave at 121 °C for 20 minutes.

After that, 100 mg of brine shrimp's eggs were added into the Petri dish containing 10 ml of artificial saline water solution (34 g of sea salt/ 1000 ml of distilled water) and allow it for hatching for

24-36 hrs with warm temperature by providing the 60W light source. After hatching, active nauplii were separated from the shells and 50 shrimp's nauplii per well were transferred to 24 well tissue culture plate with each well containing the total volume of 2 ml. Using the stem of the pipette the nauplii was counted macroscopically in a lighted background using stereozoom microscope (Meyer *et al*., 1982). To the well containing shrimp's nauplii, the ustiloxin was added at the concentrations of 0.2%, 0.4%, 0.6%, 0.8% and 1% and the plate was incubated at 25-29 °C for 48-96 hrs (Solis *et al*., 1993). Three replications were maintained for each treatment. 1% Colchicine was maintained as positive control. After incubation period, the percent death at each doses and control was determined macroscopically using stereozoom microscope. Percentage death was calculated using Abbott's formula to know the LD_{50} value.

$$\text{Percent mortality} = \frac{(\text{Control} - \text{Test})}{\text{Control}} \times 100$$

Statistical analysis

A statistical analysis was carryout to determine the disease incidence caused by *U. virens* by Completely Randomized Design (CRD) with three replications each. To discover significant difference between infected and uninfected plants, ANOVA was performed on triplicate values. Duncans Multiple Range Test was used to compare the mean values of triplicates.

RESULTS AND DISCUSSION

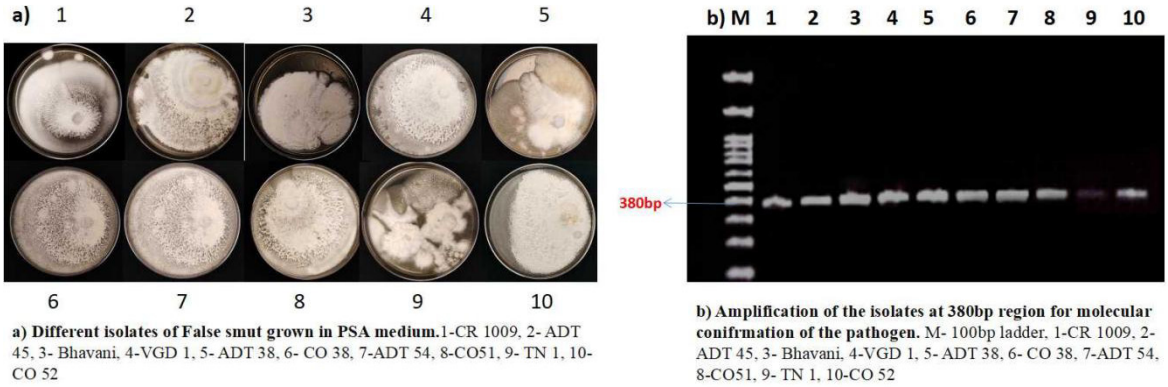
Survey and collection

Survey results revealed that the variations in the disease incidence of false smut of rice was observed in Erode, Tanjore, Perambalur and Coimbatore districts of Tamil Nadu from January-2023 to March-2023. In Erode, the disease incidence was noticed between 5 to 40% among different cultivars. In Tanjore, the disease incidence was comparatively less among the varieties with the percentage range between 7 to 21%. In Perambalur, the false smut incidence was 5.26%. In Coimbatore, the disease incidence varies from 12 to 32%, which causes reduction in yield of grains. Among the areas surveyed, higher disease incidence was recorded in the rice variety Bhavani at Bhavanisagar, Erode district with the incidence of 40% (Table 1). The infected spikelets from the regions of Erode, Tanjore, Perambalur and Coimbatore districts of Tamil Nadu revealed that the incidence varied widely from one place to another. The incidence of infected tillers was found to range between 5% and 40%, causing a substantial reduction in grain yield. Similarly, Singh *et al*., (2014) conducted survey in different regions Uttarpradesh and Tamil Nadu in which the incidence of the infected tillers range between 5-80%. Also, Navarasu *et al*., (2022) conducted survey in different regions of Tamil Nadu with the false smut incidence ranged from 1.36 to 36.36 per cent and the disease severity ranged between 1.08 to 379.23 per cent.

Isolation and characterization

The pathogen *Ustilaginoidea virens* was isolated from the infected smut balls in Potato sucrose agar (PSA) medium in which the fungus appears as small white colonies after seven days of inoculation. On observing the plate at 15th day after inoculation, the white mycelium turns yellow on maturity that is found similar to the smut balls in the field condition (Biswas, 2001). The pathogenicity of the fungus was confirmed by inoculating the pathogen in the rice variety TN1 by artificial injection of the conidial suspension to the tillers. Ashizawa *et al*., (2011) used modified method for artificial inoculation by injecting the inoculum of the fungal conidia. On observing the plants after 15th day of injection, it produces false smut balls on the panicles. The pathogen infected the ovary of the individual spikelets and transferred them to large, fluffy and velvety green balls, later these balls becomes hard after maturity. At that time, these balls looks yellow in colour and at later stages it turns olive green to greenish black. The morphological characters of the smut pathogen were observed to be creamy white with flat/ raised colony (Figure 1. (a), Table 2) having the chlamydospores at the centre that appear yellowish to green which is found to coincide with the results of Baite *et al*., (2017).

U. virens fungus was further confirmed at molecular level through Polymerase Chain Reaction (PCR) using *U. virens* specific primers, US1-5/US3-3 developed by Zhou *et al*., (2003). The products were amplified at 380 bp(Figure 1. (b))from all the collected isolates, which are specific to the false smut fungus. Ladhalakshmi *et al*. (2012) used the specific ITS primers of *U.virens* US1-5/US3-3 and US2-5/US4-3 giving amplified fragments of 380 bp and 230 bp and confirmed the identity of the false smut pathogen.



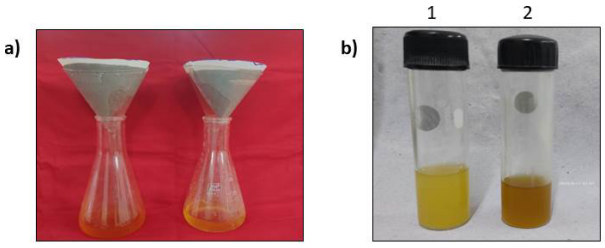
a) Different isolates of False smut grown in PSA medium. 1-CR 1009, 2- ADT 45, 3- Bhavani, 4-VGD 1, 5- ADT 38, 6- CO 38, 7-ADT 54, 8-CO51, 9- TN 1, 10-CO 52

b) Amplification of the isolates at 380bp region for molecular conifrmation of the pathogen. M- 100bp ladder, 1-CR 1009, 2-ADT 45, 3- Bhavani, 4-VGD 1, 5- ADT 38, 6- CO 38, 7-ADT 54, 8-CO51, 9- TN 1, 10-CO 52

Fig. 1. Isolation and molecular confirmation of Rice false smut (*U. virens*)

Toxin extraction and purification

The ustiloxin was extracted by using milli-Q water and 5ml of the concentrated filtrate was added in the manually packed cartridge containing HP20 resins (Figure 2(a) and 2(b)). The water-eluted fraction showed the first peak in the chromatogram and the second peak indicates 30% ethanol fraction (Fraction 3). These fractions were collected separately and condensed. Similarly Shan *et al*., (2012), used HP 20 macroporus resins during purification of ustiloxin to determine and analyse the ustiloxin A and B content in rice false smut balls by HPLC (High Performance Liquid Chromatography). Shan *et al*., (2013) developed certain methods for large-scale preparation and purification of ustiloxin A and B from smut balls. He also concluded that adsorption and desorption of SP207 and SP700 resins were found effective for the purification of Ustiloxin A and B.



a) Water extract of ustiloxin filtered through Whatman no.4 filter paper

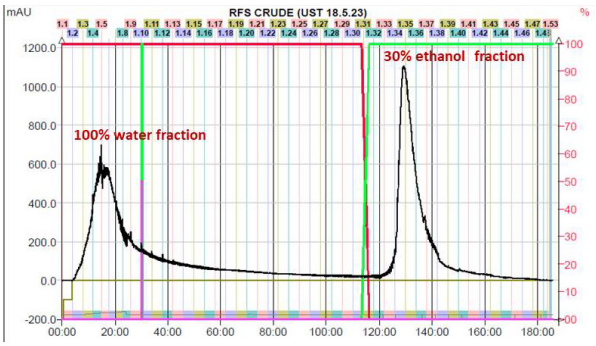
b) Fractionated crude toxin
1 - 100% water fraction,
2 – 30% ethanol fraction

Fig. 2. Extraction and purification of ustiloxin from rice false smut balls

Ustiloxin lethality bioassay on brine shrimp

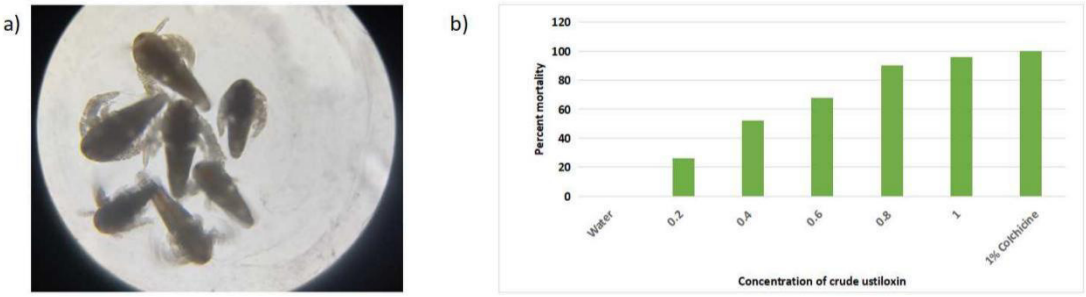
Brine shrimp (*Artemia salina*) used as a biological indicator for the mycotoxin's toxicity since the shrimp's nauplii showed susceptible to several mycotoxins (Harwig and Scott, 1971; Moretti *et al*., 2007; Durakoviæ *et al*., 2011). Several scientists validated that the effects of mycotoxin on the eggs and larvae of the brine shrimp (Favilla *et al*., 2006; Iram *et al*., 2016). Larvae were easily collected in large numbers, and their low natural mortality in medium and chloroform controls allowed for the testing of crude fungal filtrates and extracts (Harwig and Scott, 1971). In this current study, brine shrimp's (Figure.4 (a)) bioassay was used for the evaluation of biological toxicity of ustiloxin. On calculating the mortality percentage at different concentrations of ustiloxin, minimum mortality percentage was recorded at the concentration of 0.2% (26%) (Figure 4(b)). Whereas remaining all the concentration 0.4%, 0.6%, 0.8% and 1% recorded percent mortality of 52%, 68%, 90% and 96% respectively with the negative control (water) having the mortality percentage as 0. This result confirms the cytotoxicity of ustiloxin where brine shrimp is used as a model organism.

Hence, the LD50 value was calculated from brine



*The colored tags at the top of the chromatogram numbered from 1.1 to 1. 53 indicates different fractions collected in individual test tubes

Fig. 3. Chromatogram of water and ethanol fraction of ustiloxin using flash chromatography



a) Visualizing brine shrimp under **stereozoom** microscope

b) Percent mortality of brine shrimp at different concentrations of crude ustiloxin

Fig. 4. Assessment of lethal dose of ustiloxin A on brine shrimps

shrimp cytotoxicity assay was at 0.4% concentration of ustiloxin in which the percent mortality was recorded as 52% (24 nauplii survived out of 50 active nauplii added initially). Similarly Meyer *et al*., 1982 carried out cytotoxicity assay in brine shrimp by using 41 different active plant constituents from *Euphorbiaceae* and calculated the lethality percentage and LD_{50} of different constituents. Also, Solis *et al*., (1993) using 21 pharmacologically active agents and 4 quassinoids reported the LD_{50} value against brine shrimp.

CONCLUSION

Survey during first quarter of 2023 revealed that the higher incidence of rice false smut disease in bhavani variety (40%) of rice cultivated at Bhavanisagar, Erode was observed and the pathogen was isolated from false smut balls collected from different rice growing areas of Tamil Nadu. The isolates were confirmed by PCR using the specific ITS primers US 1-5/US 3-3 which amplified at 380bp for the rice false smut pathogen *Ustilaginoidea virens.* The toxin extracted from false smut balls and purified through HP 20 macroporous resins was used to analyse the ustiloxin cytotoxicity by Brine shrimp (*Artemia salina*) assay which revealed that the 0.4% of ustiloxin was confirmed as the LD_{50} concentration for brine shrimp. Therefore, we conclude that the smut balls when falls on the surface-water ecosystem of rice becomes deleterious to aquatic organisms. This study, for the first time, provided evidence that ustiloxins could be deleterious to aquatic organism of brine shrimp. This is the preliminary study to show the impact of ustiloxin on aquatic organisms of surface water in rice field. Further research is required to evaluate the impact of ustiloxin in other aquatic organisms in rice eco-system.

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Conflict of Interest : Nil

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