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Antibacterial Activities of Oregano-N-acetyl cysteine Nanocomposite against Multidrug-resistant *Riemerella anatipestifer* Isolated from Ducks

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Abstract

Riemerella anatipestifer (*R. anatipestifer*) considered a highly epizootic pathogen, causes (duck septicemia) resulting in significant ducklings' mortality and surviving birds may grow poorly leading to significant worldwide economic losses in the duck industry. As emerging of extensive multidrug resistance strains of *R. anatipestifer*, alternative treatment is of great concern for *R. anatipestifer* control. During (January - August 2020), different outbreaks in 20 suspected field duck farms, and 6 *R. anatipestifer* isolates which were confirmed by conventional PCR technique underwent an in vitro study. The isolates were tested against 17 antimicrobial agents (widely used in the poultry industry) to assess their antimicrobial resistance resulting that all tested strains being multidrug-resistant. The obtained antimicrobial resistance index (AMRI) was 0.63±0.07, a very high value. So, the alternative treatment seemed to be a valuable tool for *R. anatipestifer* control. Oregano nanoemulsion and its nanocomposite were characterized by TEM and the nano-size were 23.46 and 37.13 nm, respectively, and with a stable state, zeta potentials were 18.5±5.11, 10.031±5.11, the PDI were 0.236, 0.467 and IC50 is > 100 µg/ml and IC50 is 28.13 µg/ml, respectively). The study concluded that the tested *R. anatipestifer* strains are extensively multidrug-resistant and its control requires an alternative interference other than the antibiotics. Oregano- N-acetyl cysteine nanocomposite shows promising high activity against *R. anatipestifer*, which to be recommended.

KEYWORDS
R. anatipestifer, AMRI, Oregano- N-acetyl cysteine, Nanoalternative medicine.

INTRODUCTION

R. anatipestifer is among the germs that do ducks the most harm (Fernandez *et al*., 2018), and goose (Hess *et al*., 2013) causing great economic losses in the duck industry with different levels of fitness costs (Sun *et al*., 2019) through high mortality, and reduced growth rate (Gyuris *et al*., 2017). It affects the liver and spleen of ducklings (Flores *et al*., 2019; Flores *et al*., 2021), occasionally ducks in the breeder age group, and occasionally older ducks in the developer age group (Li *et al*., 2011; Abdelrahman *et al*., 2021) involving in the secretion of proinflammatory cytokines (Flores *et al*., 2021). Since *R. anatipestifer* without cross-protection has at least 21 known serotypes worldwide (Guo *et al*., 2017) leading to vaccination difficulties (Pathanasophon *et al*., 2002). Moreover, nowadays, *R. anatipestifer* acquired multidrug resistance as widely documented (Li *et al*., 2017; Chen *et al*., 2018; Zhu *et al*., 2018; Tzora *et al*., 2021) rather than the detection of biofilm that maximize antimicrobial resistance and interfere with the host immune system (Dermisha *et al*., 2019). AMRI is recommended to evaluate resistance virulence factor (Afunwa *et al*., 2020) since it contains multiple outdated antibiotics and originates from a source with a high risk of contamination, bacteria with a MAR index lower than 0.2 is considered to have a high index (Mthembu, 2008). So, searching for an alternative antibacterial agent other than antibiotic is of great concern to control *R. anatipestifer* infection. The present work aimed to investigate the oregano nanoemulsion and oregano-N-acetyl cysteine nano-encapsulation as alternative treatments to *R. anatipestifer*.

MATERIALS AND METHODS

Ethical Approval

Ethical committee approval was received from the Institutional Animal Care and Use Committee at the Animal Health Research Institute-Agricultural Research Center (ARC-IACUC), approval number (No: ARC-AHRI 22-35) the date it was received 12/2020 and ended 12/2022.

Samples collection

During (January - August 2020), through different outbreaks among 20 suspected field duck farms belonging to Giza Governorate, Egypt, 5 suffering birds (1-6 weeks of age) from each farm with clinical *R. anatipestifer* manifestations were collected. Lungs, liver, and tracheal swabs were aseptically collected from

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each bird sample and transported to Reference Laboratory for Veterinary Quality Control on Poultry Production (RLQP) for laboratory isolation and diagnosis.

Isolation and identification of *R. anatipestifer*: According to Tzora *et al*. (2021), samples were incubated overnight after being injected into 10 ml of Brain Heart Infusion (BHI) broth under a micro-aerophilic condition in a candled jar at 37°C. A loopful of overnight broth was inoculated onto MacConkey's agar and Blood agar plates (5% sheep blood) and further incubated for 24 hrs in micro-aerophilic condition at 37°C. Suspected *R. anatipestifer* isolates exhibited biochemical characteristics characters (catalase, oxidase, indole, and ornithine decarboxylase and gelatin liquefaction) were selected for further studies and be subjected to PCR technique (Quinn *et al*., 2011).

Confirmation of R. anatipestifer isolates by PCR technique

Following the manufacturer's instructions (Cat. No. 51304), the QIA Amp ® DNA Mini Kit (Qiagen GmbH, Hilden, Germany) was used to extract DNA from biochemically characterized isolates. The purity of DNA was checked by measuring OD values at 260 nm and 280 nm. Primers used in this study were prepared in AB Invitrogen Germany and performed as reported (Tsai *et al*., 2005). The PCR amplification product was electrophoresed in (1.5 % w/v) agarose gels (Agarose; Sigma, USA). Analysis in a Gel Documentation System (Bio-Rad, USA) to confirm the specific fragment at (665 bp). The PCR confirmed *R. anatipestifer* strains were the base of the present study.

Antimicrobial susceptibility testing

Using the antibiotic disc diffusion method, the antibiogram of the recovered isolates was assessed (Andrews, 2009; Zhong *et al*., 2009; CLSI, 2015). The positive PCR confirmed *R. anatipestifer* tested 17 antimicrobial agents commonly used in Duck farms belonging to 9 different antimicrobial groups.

MARI determination

It is computed as a/b, where a is the number of isolates that were resistant to a particular antibiotic and b is the total number of antibiotics utilized. Bacteria with a high MARI are those that come from high-risk sources of contamination where several antibiotics are employed and have a MAR value of 0.2 (Quinn *et al*., 2011).

Nanoemulsion preparation

Oregano (Origanum vulgare) was provided from the oils extracted from a unit of the National Research Center (NRC), Tween 80, N-acetyl cysteine and chitosan obtained from the Sigma-Aldrich Co., and deionized water. The prepared two nanoemulsions with/without adding N-acetyl cysteine were done in AHRI at Nanomaterials Research and Synthesis Unit. Using a homogenizer at 1000 watts, oregano oil (20%) and tween 80 were blended for five minutes. Distilled water was then gradually added to the combined oil phase (Rao and McClements, 2011).

Preparation of oregano- N-acetyl cysteine encapsulation was done by adding 2% chitosan (Bharmoria *et al*., 2021).

Characterization of nanoemulsions

Two nanomaterials were characterized using high-resolution transmission electron microscopy (HRTEM) observations with a JEM 1400F HRTEM at a 300 keV beam energy. Electrical conductivity, droplet size, surface charge (zeta potential), and size distribution (polydispersity indices, PDI) of the nanoemulsion were also measured using a Zetasizer Malvern Instrument (Corp, Malvern, UK). At Nawah Scientific Inc. (Mokatam, Cairo, Egypt), the phenolic components in oregano nanoemulsion and oregano-N-acetyl cysteine nano-encapsulation were identified by GC-MS.

Cytotoxicity assay

Sulforhodamine B (SRB) assays with various concentrations (0.01, 0.1, 1, 10, and 100 ug/ml) were used to measure cell viability. Vero: Nawah Scientific Inc. provided the green monkey cell line (Mokatam, Cairo, Egypt). The cells were kept alive in DMEM medium supplemented with 100 mg/mL streptomycin, 100 units/mL penicillin, and 10% heat-inactivated fetal bovine serum at 37°C in a humidified, 5% (v/v) CO_2 atmosphere.

Evaluation of oregano nano-emulsion and oregano-N-acetyl cysteine Nano encapsulation by using MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration)

The MIC and MBC of oregano nano-emulsion and its nano-encapsulation as antibacterial agents were done as described before (CLSI, 2013; Du *et al*., 2015). The MBC test was conducted on Blood agar plates (5% sheep blood) in micro-aerophilic conditions at 37°C for 24 hours while the MIC test was carried out for each nanocomponent in 96-well round bottom microtiter plates using conventional broth microdilution techniques.

MIC test for the oregano nano-emulsion, 100 µL of oregano stock was added to raw C (C1 to C6) then two-fold serial dilution in MHB started from raw D to the raw H, 10 µL. The bacterial inoculums were introduced to these wells at a concentration of 106 CFU/mL (for *R. anatipestifer* tested isolates R1 to R6). The raw H contains the lowest concentration, which was obtained through twofold serial dilution, while the raw C contains the highest concentration.

The N-acetyl cysteine was subjected to the same method, starting with a double serial dilution. Raw C (C7–C12) to raw H (for R. anatipastifer tested isolates R1–R6) (H 7 to H 12). The raw H contained the lowest concentration (achieved through twofold serial dilution) while the raw C contained the highest concentration (C7 to C12) (H 7 to H 12).

Raw B functioned as the positive control, whereas Raw A was retained as the negative control (medium only) (medium and bacterial inoculums). Each well of the microtiter plate received 30 L of the resazurin dye along with (0.015%) solution, which was then incubated at 37°C for 24 hours (Elshikh *et al*., 2016).

Changing the color from purple to pink indicated a positive response. The lowest concentration at which color change was noted was MIC values for the test material and bacterial strain. The lowest concentration at which no viable bacteria were detected was referred to as MBC. All assays were performed in triplicate.

RESULTS

Samples collected from six out of 20 duck farms included in the study were confirmed positively infected by the conventional method, isolates were subjected to reconfirmation.

Results of R. anatipestifer isolates by Conventional PCR technique

Screening of six isolates by PCR confirmed that all isolates

were related to *R. anatipestifer*. As all confirmed isolates exhibited the specific amplicon size of 665bp (Fig. 1).

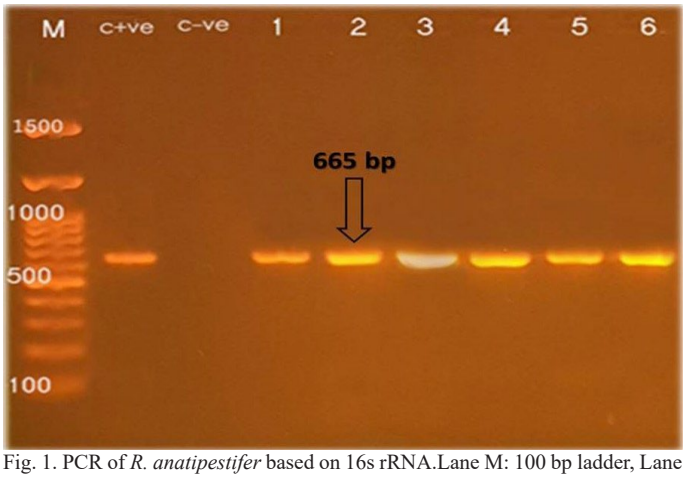

Fig. 1. PCR of *R. anatipestifer* based on 16s rRNA.Lane M: 100 bp ladder, Lane C+ve: control Positive, Lane c-ve: control Negative, Lanes 1-6: isolates with specific amplicon at 665 bp.

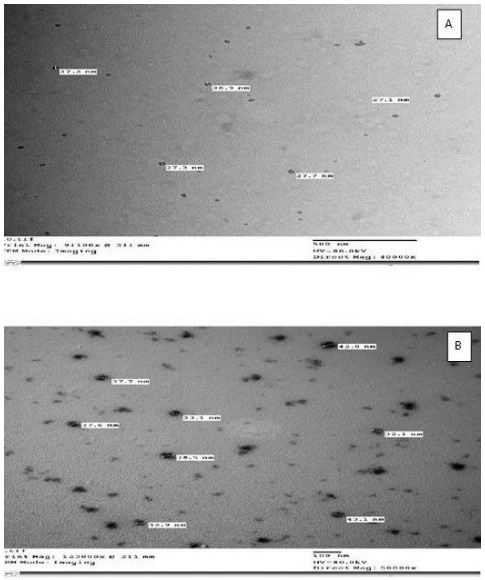

Fig. 2. a, b) TEM Oregano nanoemulsion and N-acetyl cysteine Oregano nanoemulsion.

Antibiotic susceptibility profiles of *R. anatipestifer* isolates

Data presented in Table 1 demonstrated that all studied *R. anatipestifer* isolates displayed an expanded resistance pattern to Penicillin, Ampicillin, Amoxicillin, and Rifampicin (6/6). Moreover, (5/6) of isolates were resistant to Azithromycin, Neomycin, and Tetracycline and (4/6) of isolates were resistant to Doxycycline, Norfloxacin, and Nalidixic acid. Three isolates (3/6) showed resistance to Trimethoprim/ sulphamethoxazole and Ceftriaxone while two isolates (2/6) were resistant to Levofloxacin and Erythromycin. The lowest resistance rate (1/6) was to Cefotaxime, Spectinomycin, and Ciprofloxacin. All isolates (6/6) were resistant to antimicrobial agents that belonged to more than three Antimicrobial Classes.

Oregano nanoemulsion and its nanocomposite were characterized by TEM and the nano-size were 23.46 and 37.13 nm respectively (Fig. 2 a, b), and with a stable state, zeta potentials were 18.5±5.11, 10.031±5.11, the PDI were 0.236, 0.467 and IC50 is > 100 ug/ml and IC50 is 28.13 ug/ml (Fig. 3), respectively.

When GC-Mass was analyzed the oregano nanoemulsion had many active components which were Carvacrol (2.06%),1-Dodecanamine, N, N-dimethyl- (8.19%), cis-Vaccenic acid (21.28%), n-Hexadecanoic acid (8.93%), 3-(Benzylmethylamino)-1-propanol (7.71%), 1-Tetradecanamine, N, N-dimethyl- (11.22%), Benzyl chloride (10.90%), Isochiapin B (0.72%) and 17-Pentatriacontene (2.09%) (Fig. 4). While N-acetyl cysteine Oregano nanoemulsion had many active components which were hexadecanoic acid (11.76%),10-octadecenoic acid methyl ester (6.25%), eugenol (1.96%), cis-vaccenic acid (44.41%), octadecanoic acid (9.58%), 8,11-octadecadienoic acid, methyl ester (5.70%), Isochiapin B (2.39 %), methyl 14-methylpentadecanoate (3.43%) and 17-pen-

Table 1. Antimicrobial resistance pattern and AMRI determination.

Antimicrobial Class	Antimicrobial Agent	susceptibility profile of each *R. anatipestifer* isolates					
		1*	2*	3*	4*	5*	6*
Penicillin	Penicillin G 10 µg	R	R	R	R	R	R
	Ampicillin 10 µg	R	R	R	R	R	R
	Amoxicillin10 µg	R	R	R	R	R	R
Cephalosporin (Third generation)	Ceftriaxone 30 µg	R	S	S	R	S	R
	Cefotaxime 30 µg	S	S	S	S	R	S
Macrolides	Azithromycin 15 µg	R	R	R	S	R	R
	Erythromycin 15 µg	S	S	R	S	R	S
Tetracyclines	Tetracycline 30 µg	S	R	R	R	R	R
	Doxycycline 30 µg	S	R	S	R	R	R
Fluoroquinolones	Norfloxacin 10 µg	S	R	R	S	R	R
	Levofloxacin 5 µg	S	S	S	S	R	R
	Ciprofloxacin 5 µg	R	S	S	S	S	S
Synthetic Quinolones	Nalidixic A 30 µg	S	R	R	S	R	R
Sulfonamides	Trimethoprim/sulfamethoxazole 25 µg	S	S	R	S	R	R
Aminoglycosides	Neomycin 10 µg	R	S	R	R	R	R
	Spectinomycin 100µg	S	S	S	S	S	R
Rifamycins	Rifampicin 5 µg	R	R	R	R	R	R
AMRI		0.47	0.53	0.65	0.47	0.82	0.82
		Overall (0.63±0.07)					

tatriacontene (15.33%) (Fig. 5).

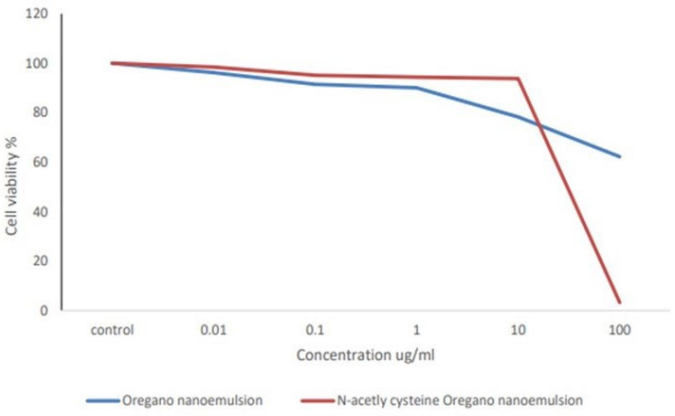

Fig. 3. Cell viability % of Oregano nanoemulsion and Oregano-N-acetyl cysteine nano-encapsulation effect on Vero cells.

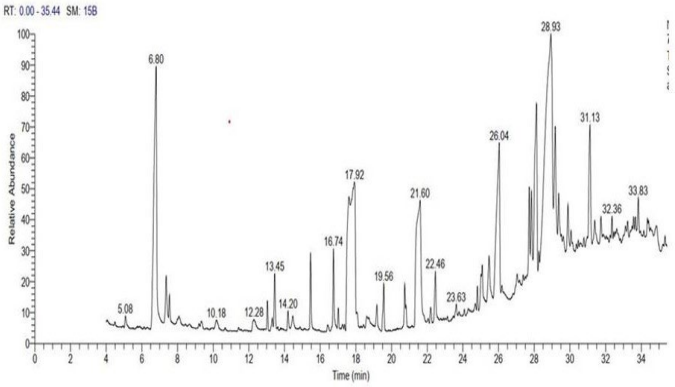

Fig. 4. GC-Mass analysis of Oregano nanoemulsion.

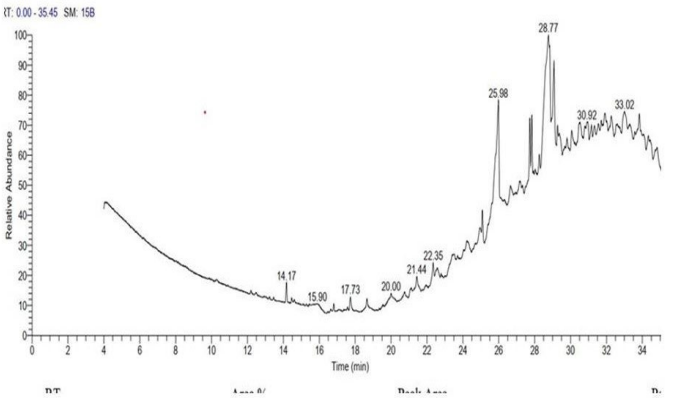

Fig. 5. GC-Mass analysis of N-acetyl cysteine Oregano nanoemulsion.

Determination of the in vitro antibacterial activity of oregano nano-emulsion and oregano-N-acetyl cysteine nano-encapsulation against the six *R. anatipestifer* isolates were expressed as MICs and MBCs (Fig. 6). The MIC values for oregano nano-emulsion were equal to 0.03125µg/mL for 2 out of 6 *R. anatipestifer* isolates; the MICs of the remaining four isolates were 0.0625 µg/mL and 0.125 µg/mL The MBC values were 2–4 times lesser than MICs (ranging from 0.125 µg/mL to 0.015625 µg/mL) in all isolates.

DISCUSSION

The biological activities of oregano essential oil (EO) are varied. Its hydrophobicity and instability, however, restrict its use in medicinal formulations. In the current study, a stable oregano EO nanoemulsion (ONE) was created to evaluate its antibacterial and anticancer properties in comparison to free (OEO) and coarse emulsion (OE) forms. Carvacrol (65.24%) and linalool (12.32%) were found to be the two main oil constituents in oregano EO after they had been hydro-distilled and analyzed using gas chromatography-mass spectrometry. The nanoemulsion with the best stability profile had droplet sizes of 88.37 nm (0.104 PDI). Pseudomonas aeruginosa was the only studied bacterial strain, in that Pseudomonas did not display antibacterial action, and its activity was considerably stronger against Staphylococcus epidermidis, E. coli, and Enterococcus faecalis (Manaa *et al*., 2022).

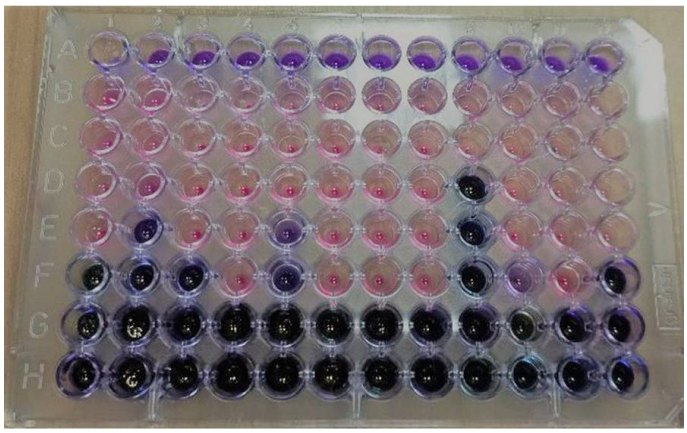

Fig. 6. Determination of minimum inhibitory concentration (MIC) by resazurin microtitre assay method for carvacrol and N-acetyl cysteine against the six *R. anatipestifer* isolates.

One of the most harmful diseases impacting duck farms globally is *R. anatipestifer*, which can be acute or chronic. Septicemia since it can use hemin from bovine and duck hemoglobin (Hb) (Flores *et al*., 2021) in outbreaks which were reported for domestic ducks and turkeys, but may affect broiler chickens (Tzora *et al*., 2021), turkeys, and other poultry (Sun *et al*., 2019) causing great economic losses. As the misuse of antibiotics for microbial infection treatment led to the emergence of multidrug resistance bacteria, *R. anatipestifer* acquired multidrug resistance pattern (Li *et al*., 2017; Chen *et al*., 2018; Zhu *et al*., 2018; Tzora *et al*., 2021).

In the present study, the etiological pathogen among the twenty duck farms outbreaks was *R. anatipestifer* which was confirmed by PCR technique and showed a very extensive multidrug resistance pattern with very high AMRI (0.63±0.07) where ≤ 0.2 is considered as a high index (Table 1). Therefore, raised the need for alternative treatment other than antibiotics and to develop new strategies (WHO, 2015) for its treatment and control.

The microdilution process is standardized, precise, straightforward, and affordable to use. The improved micro-dilution method described in this report is enhanced through the addition of resazurin dye as redox indicator oxygen reductases are produced by living, active bacteria and convert the fluorescent resorufin (pink) from the non-fluorescent resazurin (blue), which can then be further reduced to hydroresorufin (McNicholl *et al*., 2007). In the current investigation, resazurin dye was utilized as an indicator to measure bacterial growth, which was shown by a shift in color from purple to pink in each well (or colorless).

The MIC values for oregano-N-acetyl cysteine Nano encapsulation were equal to 0.03125µg/mL for 3 out of 6 isolates; the MICs of the remaining three isolates were equal to 0.0625 µg/mL (2/ 6) and 0.125 µg/mL (1/6).

The MBC values were 2–4 times lesser than MICs (from 0.0625µg/mL to 0.015625µg/mL) in all *R. anatipestifer* isolate.

The *in vitro* N-acetyl cysteine antimicrobial activity is documented (Ivanovic *et al*., 2012), even virulent pathogens such as TB (Amaral *et al*., 2016), St. pyrogens (Wijesundara *et al*., 2021), uropathogenic E. coli (Khan *et al*., 2017), or multidrug-resistant MRSA (Abed *et al*., 2021).

The promising obtained remarks were carvacrol EO can alter the expression of their genes and their phenotypic characteris-

tics, even in sub-lethal levels (Abed *et al*., 2021).

To the best of the authors' knowledge, the present study is the first available study about its antimicrobial activity against *R. anatipestifer* with promising results recommending further study for *in vivo* trials.

CONCLUSION

It can be concluded that using oregano-N-acetyl cysteine nano-encapsulation at 0.0625 µg/mL is very effective in the treatment of *R. anatipestifer* infection.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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