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lsolation and characterization of *E. coli* and *Salmonella* species from diarrhea patients visiting University of Nigeria Teaching Hospital (UNTH), Enugu State University Teaching Hospital (ESUTH) and National Orthopaedic Hospital Enugu (NOHE) in Enugu State.

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ABSTRACT

E. coli and *Salmonella* species from diarrhea patients visiting University of Nigeria Teaching Hospital (UNTH), Enugu State University Teaching Hospital (ESUTH) and National Orthopaedic Hospital Enugu (NOHE) in Enugu State were analyzed. The bacteria isolates were identified using the morphological appearance (macroscopy) of their colonies, their gram stain reaction (microscopy) and confirmatory biochemical and sugar fermentation tests. The result showed that in ESUTH and UNTH, the distribution of *Escherichia coli* and *Salmonella typhi* isolates were more in the in-patients across the various age groups than the out-patients. The distribution of isolates across age groups in NOHE showed a higher trend among out-patients than in-patients. In conclusion, from this present study, *Escherichia coli* and *Salmonella* spp. were isolated among pediatric age groups (1-10) with prevalence rate of 67 % and 13 % respectively.

Keywords: lsolation, characterization, *E. coli*, *Salmonella* and diarrhea.

INTRODUCTION

The rapid emergence of resistant bacteria is occurring worldwide, endangering the efficacy of antibiotics, which have transformed medicine and saved millions of lives [1,2,3,4]. Many decades after the first patients were treated with antibiotics, bacterial infections have again become a threat [5,6,7,8]. The antibiotic resistance crisis has been attributed to the overuse and misuse of these medications, as well as a lack of new drug development by the pharmaceutical industry due to reduced economic incentives and challenging regulatory requirements [9,10,11,12]. The Centers for Disease Control and Prevention (CDC) has classified a number of bacteria as presenting urgent, serious, and concerning threats, many of which are already responsible for placing a substantial clinical and financial burden on the U.S. health care system, patients, and their families [13,14,15]. Coordinated efforts to implement new policies, renew research efforts, and pursue steps to manage the crisis are greatly needed [16,17]. The major cause of this crisis is the indiscriminate and widespread use of antibiotics, especially the beta-lactams (antibiotics containing beta-lactam ring), in prophylaxis and treatment of bacterial diseases [18]. The misuse and abuse of β-lactam antibiotics bought over the counter without doctor's prescription has led to antibiotic selective pressure and development of resistance to these drugs by most bacteria, particularly *E. coli* and *Salmonella* of which β-lactamase production remains the most important contributing factor to this resistance [19].

Aim and Objective of the study

The aim of this research was to isolate and characterize *E. coli* and *Salmonella* species from diarrhea patients visiting University of Nigeria Teaching Hospital (UNTH), Enugu State University Teaching Hospital (ESUTH) and National Orthopaedic Hospital Enugu (NOHE) in Enugu State.

MATERIALS AND METHODS

Study Area

Enugu, usually referred to as Enugu State to distinguish it from the city of Enugu is a state in Southeastern Nigeria, created in 1991 from part of the old Anambra State. Its capital and largest city is Enugu, from which the state derives its name. Enugu State is one of the states in eastern part of Nigeria located at the foot of the Udi Plateau. The State shares borders with Abia State and Imo State to the south, Ebonyi State to the east, Benue State to the Northeast, Kogi State to the Northwest and Anambra State to the West. The principal cities in the state are Enugu, Nsukka, Agbani and Awgu. It has a total population of 3,267,837 individuals according to 2006 census and population density of 460/km2 with coordinates of 60 30'N 70 30'E; total land area of about 7,161 km2. Economically, the state is predominantly rural and agrarian, with substantial proportion of its working population engaged in farming, although trading (18.8 %) and civil services (12.9 %) are also important. In the urban areas trading is the dominant occupation, followed by civil services. A small proportion of the population is also engaged in manufacturing activities, with the most pronounced, among them located in Enugu, Oji, Ohebedim and Nsukka. It has some tertiary health institutions and numerous private hospitals and clinics. In the state, there are seven District Hospitals in Enugu – Urban, Udi, Agbani, Awgu, Ikem, Enugu Ezike, and Nsukka and at least one health centre or cottage hospital in each of the 17 Local Government Area of the State (William, 2008; National Beareu of Statistics, 2010). The study areas were three tertiary health institutions in Enugu State. The study area covered the three senatorial zones (Enugu East, Enugu North and Enugu West) of the state and the hospitals serve as a major referral points in the state.

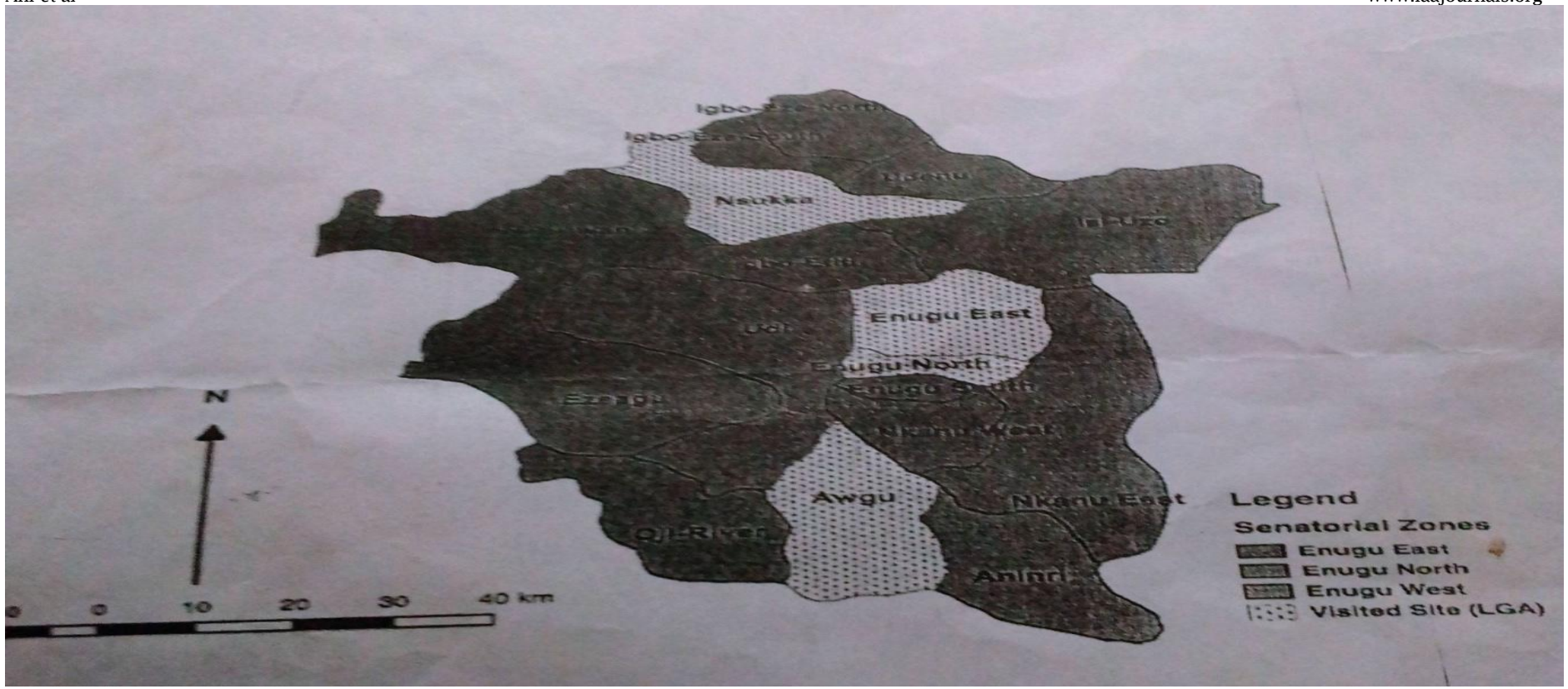

Figure 1: Map of Enugu State showing the three senatorial zones and the L.G.As of the study sites (Ezeigwo, 2009).

Study Population

The samples used were sourced from these tertiary hospitals in Enugu State. Parklane Teaching Hospital, National Orthopeadic Hospital and University of Nigeria Teaching Hospital.

Study Period

The study was conducted over a period of one and half years (from June 2017 to January 2019) and four hundred and fifty (450) stool samples from Out-patients and In-patients Departments of the hospitals were examined using the laboratory sections of the hospitals as the sampling points.

Ethical Clearance

Ethical clearance was granted by the Joint Committee on Human Research Publications and Ethics of the hospitals – vide: UNTH/CSA/329/VOL.5, ESUTHP/C-MAC/RA/034/Vol. II and NOHE S/313/IX/963.

Determination of Sample Size

The sample size was calculated using Daniel's standard size determination technique according to the following equation [3]. The formula $n = (Z_1 - \alpha)^2 (P (1-P)/d^3$ was used to calculate the sample size, where P is the estimated proportion and d is the desired precision (Suresh and Chandrashekara, 2012). The sample size was calculated using an approximate prevalence rate of 50%, confidence interval of 95 % and precision of 5 %. The value of $Z_1-\alpha$ at 5% level of significance was 1.96. Using the formula with provision for 10 % attrition, a sample size of 450 (150 from each hospital and from patients with enteric diseases) was used.

Sterilization of Glassware/Media/Equipment

All glass wares used were washed and sterilized in hot air oven at temperature of 160^0C for 1hr according to the standard method described by [9]. Culture media were reconstituted and sterilized at 121^0 C for 15 mins using an autoclave. Metallic equipment (spatula, wire loop, Bunsen burner, etc) were sterilized under flame for 2-5 mins and the work bench was sterilized using 70 % ethanol.

Media Preparation

All media used include: MacConkey agar, Nutrient agar, Mueller Hinton agar, Nutrient broth, Cystine-lactose electrolyte-deficient agar (CLED) and *Salmonella Shigella* Agar.

Microbial Identification and Characterization

The bacteria isolates were identified using the morphological appearance (macroscopy) of their colonies, their Gram stain reaction (microscopy), and confirmatory biochemical and sugar fermentation tests.

Gram Staining

Smears of the discrete colonies were made by placing a drop of distilled water on clean grease free slides. This was air dried and the smear heat fixed by passing it three times over a Bunsen burner flame. These were covered with Crystal violet stain for 60 seconds and rinsed with running water. Thereafter, Gram's iodine was poured on the smear as a mordant and allowed to stay for 60 seconds and rinsed off with clean water. They were then rapidly decolorized for few seconds with acetone and immediately washed off with clean water. They were counter stained with Safranin stain for 30 seconds and washed off with clean water. The back of the slides was drained and allowed to air dry, after which it was examined under the oil immersion objective microscope (X100) for gram characteristics of the organisms. The organism that retained the purple coloration was taken as Gram-positive while those that retained the red coloration were taken as Gram-negative [4].

Biochemical Tests

The following biochemical tests were carried out; Catalase, Urease, oxidase, Indole, Motility, Voges-Proskauer (VP), Methyl red, Sugar fermentation test and Citrate utilization tests were conducted to confirm the results obtained from the macroscopic and microscopic examination of the isolates.

Standardization of Test Organisms

All bacterial isolates were standardized before use. A loopful of the test bacteria from the nutrient agar slant was inoculated into 5 ml nutrient broth in a sterile test tube and incubated. A loopful of the nutrient broth culture was then diluted with 5 ml sterile water to obtain microbial population of 10^5colony forming unit per milliliter (CFU/ml), equivalent to 0.5 McFarland standards by incubating at temperature of 37^0C for 3 hours [9].

RESULTS

The result shown in Table 1 represents the summary of the cultural, microscopic, biochemical and sugar fermentation tests for the characterization of *Escherichia coli* and *Salmonella typhi* from stool sample.

Table 1: Identification and characterization of *Escherichia coli* and *Salmonella typhi* from Stool Samples of the Three Hospitals in Enugu State.

Colony morphology	Gram Stain		Biochemical Test								Sugar Fermentation				Suspected Organism
	Gram Reaction	Cell Morphology	Cat.	Oxi.	Cit.	M.R	VP	Ind.	Ure.	Mot	Glu.	Lac.	Mal.	Suc	
White, small, smooth, circular colonies	-	Rods	+	-	-	+	-	+	-	+	+	+	-	v	*Escherichia coli*
Pale, smooth, yellow, circular colonies	-	Rods	+	-	+	+	-	+	+	+	+	-	+	-	*Salmonella typhi*

Key: cat-catalase, oxi- oxidase, cit- citrate, M,R- methyl red, VP-voges proskaeur, ure- urease, Ind- Indole, Mot- Motility, Glu- Glucose, Lac- Lactose, Mal-Maltose and Suc-Sucrose.

Frequency of *Escherichia coli* and *Salmonella typhi* isolates from stool samples according to hospitals is presented in Table 3. A total of 450 stool samples were collected, out of these samples, 147 (82%), 60 (67%) and 147 (82%) were isolated from Enugu State Teaching Hospital, National Orthopedic Hospital Enugu and University of Nigeria Teaching Hospital respectively.

Table 2: Frequency of *Escherichia coli* and *Salmonella typhi* isolates from stool samples according to hospitals

Hospitals	No of samples collected	Percentage Frequency
ESUTH	180	147 (82%)
NOHE	90	60 (67%)
UNTH	180	147 (82%)
TOTAL	450	354 (231%)

Keys: ESUTH: Enugu State University Teaching Hospitals, UNTH: University of Nigeria Teaching Hospital Ituku Ozalla, NOHE: National Orthopedic Hospital, Enugu.

The percentage distribution of the 354 *E.coli* and *S.typhi* isolated based on the hospital sourced is presented in Table 4. UNTH had the highest prevalence of *Escherichia coli* with 147(48 %), followed by 117(38%) from ESUTH and least in NOHE with 45(15 %). ESUTH also had the highest prevalence of *Salmonella typhi* with 30(67%), followed by NOHE with 15(33%) and none in UNTH.

Table 3: Percentage (%) distribution of *Escherichia coli* and *Salmonella typhi* isolates according to hospital.

Organisms	N	NOHE (%)	ESUTH (%)	UNTH %
Escherichia coli	309	45 (15)	117 (38)	147 (48)
Salmonella typhi	45	15 (33)	30 (67)	- (0)
Total	354 (100)	60 (48)	147 (105)	147 (48)

Key: NOHE: National Orthopaedic Hospital, Enugu, ESUTH: Enugu State University Teaching Hospital, UNTH: University of Nigeria Teaching Hospital, N= Number of isolated organism.

Frequency of *Escherichia coli* and *Salmonella typhi* isolates from different hospitals according to gender is presented in Table 5. A Total of 107 (73%), 97 (83%) and 30 (67%) from females and 40 (27%) and 20 (17%) and 15 (33%) males of *Escherichia coli* were isolated in UNTH, ESUTH and NOHE; 20(67%), 00(0%) and 10(67%) from females and 10(33%), 00(0%) and 15(100%) males of *Salmonella typhi* were isolated in ESUTH, UNTH and NOHE respectively.

Table 4: Percentage Frequency of isolation of *Escherichia coli* and *Salmonella typhi* from different hospital according to gender.

Gender	UNTH (%)		ESUTH (%)		NOHE (%)	
	E.coli	*S.typhi*	*E.coli*	*S.typhi*	*E. coli*	*S. typhi*
Females	170(73)	00(0)	97(83)	20(67)	30(67)	10(67)
Males	40(27)	00(0)	20(17)	10(33)	15(33)	5(33)
Total	147(100)	00(0)	117(100)	30(100)	45(100)	15(100)

Key: ESUTH: Enugu State University Teaching Hospital, UNTH: University of Nigeria Teaching Hospital, NOHE: National Orthopedic Hospital, Enugu, *E.coli=Escherichia coli*, *S.typhi= Salmonella typh*

Frequency of *Escherichia coli* and *Salmonella typhi* isolates from different hospitals according to type of patients (In-patients and Out-patients) is presented in Table 6. Out of 170 *Escherichia coli* and 17 *Salmonella typhi* isolated from In-patients across the hospitals, ESUTH, UNTH and NOHE for *E.coli* had 70 (41%), 90 (53%) and 10 (6%) while 17 (100%), 00 (0%) and 00 (0%) were for *Salmonella typhi* respectively. *E.coli* isolation from out patients showed the highest frequency in UNTH with 57 (41%) followed by 47 (34%) from ESUTH and 35 (25%) from NOHE while Salmonella typhi showed highest frequency in NOHE with 15 (54%) followed by 13 (46%) from ESUTH and 00 (0%) from UNTH respectively.

Table 5: Frequency of Isolation of *Escherichia coli* and *Salmonella typhi* isolates from different hospital according to type of patients (In-patients and Out-patients).

Type of patients	No of Samples Examined		ESUTH (%)		UNTH(%)		NOHE(%)	
	E.coli	*S. typhi*	*E.coli*	*S.typhi*	*E. coli*	*S. typhi*	*E.coli*	*S. typhi*
In-Patients	170	17	70(41)	17(100)	90(53)	00(0)	10(6)	00(0)
Out-Patients	139	28	47(34)	13(46)	57(41)	00(0)	35(26)	15(54)
Total	309	45	117(75)	30(146)	147(94)	00(0)	45(31)	15(54)

Key: ESUTH: Enugu State University Teaching Hospital, UNTH: University of Nigeria Teaching Hospital, NOHE: National Orthopedic Hospital, Enugu, *E.coli=Escherichia coli*, *S.typhi= Salmonella typhi.*

Tables 6-8 Show the percentage distribution of *Escherichia coli* and *Salmonella typhi* isolates from the various hospitals according to type of patients (In-patients and Out-patients) and age groups.

In ESUTH and UNTH, the distribution of *Escherichia coli* and *Salmonella typhi* isolates were more in the In-patients across the various age groups than the out-patients. The distribution of isolates across age groups in NOHE showed a higher trend among out-patients than In-patients.

Table 6: Percentage Distribution of *Escherichia.coli* and *Salmonella typhi* from ESUTH according to type of patients and age groups.

Age in	In-Patient		Out-Patient	
Years	*E.coli* (%)	*S. typhi* (%)	*E.coli* (%)	*S.typhi*. (%)
1 – 10	15 (3)	3 (10)	12 (10)	2 (7)
11 – 20	13 (11)	2 (7)	10 (9)	3 (10)
21 – 30	12 (10)	4 (18)	8 (7)	1 (3)
31 – 40	14 (12)	2 (7)	5 (4)	3 (10)
41 – 50	7 (5)	3 (10)	7 (6)	2 (7)
51 – Above	9 (8)	3 (10)	5 (4)	2 (7)
Total	70 (60)	17 (51)	47 (40)	13 (44)

Key: *E.coli=Escherichia coli, S.typhi= Salmonella typhi*.

Table 7: Percentage Distribution of *Escherichia coli* and *Salmonella typhi* isolates from UNTH according to type of patients and age groups.

Age in	In-Patient		Out-Patient	
Years	*E.coli* (%)	*S.typhi* (%)	*E.coli* (%)	*S.typhi*. (%)
1 – 10	16 (11)	0 (00)	10 (7)	0 (00)
11 – 20	15 (10)	0 (00)	12 (8)	0 (00)
21 – 30	19 (13)	0 (00)	8 (5)	0 (00)
31 – 40	21 (14)	0 (00)	12 (8)	0 (00)
41 – 50	9 (6)	0 (00)	10 (7)	0 (00)
51 – Above	10 (7)	0 (00)	5 (3)	0 (00)
Total	90 (60)	0 (00)	57 (38)	0 (00)

Key: *E.coli=Escherichia coli, S. typhi= Salmonella typhi*.

Table 8: Percentage (%) Distribution of *Escherichia coli* and *Salmonella typhi* isolates from NOHE according to type of patients and age groups.

Age in Years	In-Patient		Out-Patient	
	E.coli (%)	*S.typhi*(%)	*E.coli* (%)	*S.typhi* (%)
1 – 10	2 (4)	0 (00)	5 (11)	3 (20)
11 – 20	1 (2)	0 (00)	7 (16)	1 (6)
21 – 30	2 (4)	0 (00)	6 (13)	4 (27)
31 – 40	2 (4)	0 (00)	7 (16)	4 (27)
41 – 50	1 (2)	0 (00)	4 (9)	1 (6)
51 – Above	2 (4)	0 (00)	6 (13)	2 (13)
Total	10 (20)	0 (00)	35 (78)	15 (99)

Key: *E.coli=Escherichia coli, S. typhi= Salmonella typhi*.

DISCUSSION

Isolation and characterization of *E. coli* and *Salmonella* species from diarrhea patients in tertiary hospitals in Enugu State

Bacteria-associated diarrheal diseases are a major public health problem in developing countries where illiteracy, poverty, overcrowding, poor sanitation, and unsafe drinking water supply are common [10]. In this study, we highlighted a lot of prevalence factors on the risk of diarrhea associated with *Enterobactericea*. Improper faecal disposal as well as lack of clean water lead to contamination of groundwater especially in areas where water filtration or purification processes are not practiced. This can help in the wide spread of pathogens. In this study *E. coli* accounted for the highest prevalence with 87.2 % while *Salmonella typhi* had a lower frequency of 12.8 % respectively. This is in agreement with the work of [14] that an 88 % and 12 % faecal specimen yielded *E. coli* and *Salmonella sp.* respectively of diarrheal patient samples. This implies that there is co-infection among the study participants. It has been reported that co-infections with multiple enteric pathogens occur mainly in zones with poor quality of food, drinking water and poor sanitary conditions in the environment [6].

The prevalence rate observed in the present study was also lower than 31.5% reported among internally displaced persons in Nassarawa State [13]. The lower prevalence can be attributed to the fact that children in the current study lived in regular homes in contrast to the usually poor hygienic conditions often found in camps for displaced persons. The present study recorded a higher prevalence than 2.7% prevalence reported in a hospital-based study in Jos University Teaching Hospital (JUTH) [9]. The higher prevalence obtained in the present study may have resulted from inclusion of infants aged less than six months, a group excluded in the Jos study. Considering that diarrhoea is very common in this group of young infants, it is not surprising that the current study recorded a higher prevalence than the Jos study. The present study recorded a lower prevalence rate compared to the study in a hospital-based study in Accra, Ghana [10], which reported 28.0% prevalence of acute diarrhoea among under-five children. The present study

also recorded a lower prevalence compared to a hospital-based study in Soweto, South Africa [7], which reported 21% prevalence. The likely reason the present study reported a lower prevalence compared to the studies in Ghana and South Africa may be due to fact that the studies were carried out in cottage hospitals primarily caring for children with diarrhoea and malnutrition. The current study did not report the presence of stool isolates such as *Campylobacter* species as reported by researchers in Enugu [5,8,10]. This may be attributed to the culture media that was used in the current study which does not allow for growth of such isolates. The high yield of bacterial isolates in stool could be due to the collection of samples from potty/ diapers of participants and contaminants from urine which cannot be completely ruled out. Conversely, the use of three culture media contributes to high yield of the stool isolates obtained in the current study.

CONCLUSION

In conclusion, from this present study, *Escherichia coli* and *Salmonella* spp. were frequently isolated among pediatric age groups (1-10) with prevalence rate of 67 % and 13 % respectively.

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