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Full Length Research Paper

Antimicrobial resistance and plasmid profiles of *Aeromonas hydrophila* isolated from River Njoro, Kenya

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The purpose of this study was to investigate the presence of *Aeromonas hydrophila* at commonly used water collection points on the River Njoro and to determine the *in-vitro* antimicrobial susceptibility and plasmid profiles of isolates. In total, 126 samples were collected and 36.5% of them were positive for *A. hydrophila*. The *A. hydrophila* were recovered on membrane filters, cultured on Trypticase Soy agar, Bile aesculin agar and Aeromonas Medium agar. They were further characterized using cytochrome oxidase and API 20E tests. Detection of drug susceptibility was determined using modified disc diffusion method to ampicillin (25 µg), cefaclor (30 µg), ceftizoxime (30 µg), cefixime (5 µg), cefazidime (30 µg), gentamicin (200 µg), streptomycin (25 µg), chloramphenicol (50 µg), nalidixic acid (30 µg) and ciprofloxacin (1 µg). Most of the isolates showed multi-drug resistance to two or more antibiotics. Chloramphenicol, nalidixic acid, ciprofloxacin, cefazidime and cefixime were the most sensitive drugs with 100% efficacy whereas ampicillin, cefaclor and streptomycin were the most resistant drugs having 100, 67 and 50 resistance, respectively. There was low resistance against ceftizoxime (16.7%) and gentamicin (23.3%). These results indicates that all *A. hydrophila* isolated from River Njoro had complete resistance to ampicillin and showed variable resistance to cefaclor, streptomycin, gentamycin and ceftizoxime. R-plasmids were extracted from multi-drug resistance strains and separated by agarose gel (0.8%) electrophoresis for profiling. Plasmid profiling revealed that most of the multi-drug resistant isolates contained one plasmid of 21.0 kb. Although some strains exhibited different antimicrobial resistance patterns, all of their plasmids were of the same size (21.0 kb). However, there were no plasmids in the antimicrobial sensitive isolates. This study also indicates that plasmid 21.0 kb is common in *A. hydrophila* and is important for antimicrobial resistance and virulence. Further studies are required to ascertain the role of this plasmid as a virulence marker.

Key words: *Aeromonas hydrophila*, antimicrobial resistance, plasmid profile.

INTRODUCTION

Species of *Aeromonas* are found in a wide and diverse range of habitats ranging from commercially produced food products to fresh/brackish water, ground water, raw sewage, both polluted and unpolluted streams and rivers (Chang and Bolton, 1987; Hanninen et al., 1997; Fernandez et al., 2000; Erova et al., 2006; Silas et al., 2011). These bacteria have also been isolated from chlorinated and unchlorinated water and bottled mineral water, which shows that they are able to withstand long periods of nutrient limitation (Janda, 1990; Kuhn et al., 1997). Other studies have demonstrated that the presence of *Aeromo-*

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nas species in drinking water is a potential risk, since these microorganisms can produce a wide range of virulence factors (Fox et al., 1990; Messi et al., 2002). These factors include fimbriae, siderophores, capsule, toxins, quorum sensing, biofilm formation, adhesins, S-layer, lipopolysaccharides, type II and III secretion system among other (Janda, 1990; Kuhn et al., 1997; Coloe et al., 2007). *Aeromonas* species are responsible for conditions such as severe gastroenteritis, traveller's diarrhoea, haemolytic-uremic syndrome, wound infections, cellulitis, myonecrosis, ecthyma gangrenosum, septicaemia and urinary tract infections (Fox et al., 1990; Hanninen et al., 1997; Kuhn et al., 1997; Coloe et al., 2007).

Food and water-borne outbreaks of *A. hydrophila* have been documented from a number of countries (Fox et al., 1990; Poirier et al., 1993; Chang et al., 2007; Coloe et al., 2007). The difficulties in the treatment of gastrointestinal diseases due to *A. Hydrophila*, have been compounded by the continued emergence of antimicrobial resistance to a growing number of antimicrobial agents such as tetracycline, gentamycin, norfloxacin, ampicillin, amoxicillin, streptomycin and trimethoprim among others (Chang and Bolton, 1987; Erova et al., 2006; Abulhamd, 2009). According to Abulhamd (2009) and Ko et al. (1998), infections caused by *A. hydrophila* have become a significant public health problem world-wide with the evolution of multi-resistance antimicrobial plasmid genes. The transferable nature of the gene clusters encoding high level multiple antibiotic resistances in bacteria environment has caused concerns particularly in transmission of resistant isolates between people and consumption of food from animals that have received antimicrobial agents (Mikhail et al., 1990; Power, 2006).

Aeromonas species isolated from water have been found to be susceptible to most Gram negative antibiotics, though resistances have been demonstrated to occur against chloramphenicol, tetracycline, co-trimoxazole, ampicillin and sulfonamide/trimethoprim (CDC, 2003). These bacteria have been shown to have capacity to receive and transfer antibiotic resistance genes to other Gram negative bacteria (Fox et al., 1990; Silas et al., 2011). River Njoro is the major source of water for domestic, industrial and agricultural use for the surrounding community including Nakuru town, Njoro town, Kenya Agricultural Research Institute (KARI), Njoro Canning, Egerton University, Njokerio and Ndeffo town. Effluents from Njoro Canning, untreated wastes from Njokerio abattoir and KARI are released into the river in considerable amounts. During the rainy season, pollutants from the farms along the river and poorly disposed human waste are washed off to the river. This poses risks to health of communities abstracting water from River Njoro for domestic purposes. Currently there is no existing epidemiological data for gastrointestinal infections causing diarrhoea directly attributed to *A. hydrophila* or any *Aeromonas* spp in the Njoro region. Even though Njoro residents have been faced with increasing cases of diarrhoe of known and unknown aetiologies, clinical bacterial isolates have not been identified to species level and patients are only treated empirically (NPGH, 2009).

This study was conducted to investigate the presence of *A. hydrophila* in River Njoro and to determine their antimicrobial resistance. The isolates were investigated for the presence of R-plasmid (21.0 kb) which is a stable plasmid of *A. hydrophila* important for virulence and pathogenicity (Mazumder, 2009).

MATERIALS AND METHODS

Sample collection

A total of 126 samples were collected from 6 sampling sites of River Njoro in Kenya, namely; Logoman, Neesuit, Beeston, Egerton, Njoro and Ngata (Figure 1). The sites were selected on the criteria that they were commonly used communal points of water collection for domestic use (washing, cleaning, cooking and bathing) and watering livestock. All sampling sites were in the immediate vicinity of population concentrations with no piped water and all sites showed evidence of heavy domestic and agricultural use.

Samples were collected to the volume of 100 ml in sterile glass bottles. All samples were immediately sealed, labelled, placed in dark containers and processed within 4 h of collection to ensure sample integrity. Water samples were pre-enriched in buffered peptone water (BPW) and incubated at 37°C for 3 h then filtered through 0.22 µm cellulose nitrate membrane (Whatman GmbH, Germany). The membranes were pre-sterilized by manufacturers through gamma irradiation.

Microbiological identification of *A. hydrophila*

Membrane filters were then aseptically removed from the membrane holder, inverted and cultured on Aeromonas Medium Base (RYAN) agar (Oxoid, UK), then subcultured on Bile Aesculin agar (Oxoid, UK-Basingstoke) and incubated for 24 h at 37°C.

Aeromonas agar was impregnated with ampicillin (5.0 µg/ml) as an additional selective agent to screen for and isolate *Aeromonas* species. Isolated colonies were confirmed as *A. hydrophila*–positive using API-20E (BioMerieux, Durham, North Carolina, USA) as described by Saad et al. (2005).

Antimicrobial susceptibility testing

A. hydrophila was tested against 10 antimicrobial agents. The antimicrobial agents were split between those commonly used in the region (gentamicin (200 µg), streptomycin (25 µg), chloramphenicol (50 µg), ampicillin (25 µg), nalidixic acid (30 µg) and ciprofloxacin (1 µg) and the ones that are not administered in the area (cefaclor (30 µg), cefixime (5 µg), ceftazidime (30 µg) and ceftizoxime (30 µg) (Oxoid laboratories, Basingstoke-UK).

Disc diffusion was employed in determining sensitivity of pure cultures of *A. hydrophila* using Muller Hinton (Himedia Laboratories-Mumbai) agar as described by Clinical and Laboratory standards Institute (CLSI, 2006).

The bacteria inocula were adjusted in sterile 0.9% saline to 0.5 Macfarland turbidity standards (10^8 cfu/ml) and spread on Mueller Hinton agar. Antimicrobial discs were applied to the plate 15 min after inoculation. All the isolates were subjected to antimicrobial susceptibility testing using the above mentioned drugs. Inhibition zone sizes were interpreted using standard recommendations of Clinical and Laboratory Standards Institute (CLSI, 2006).

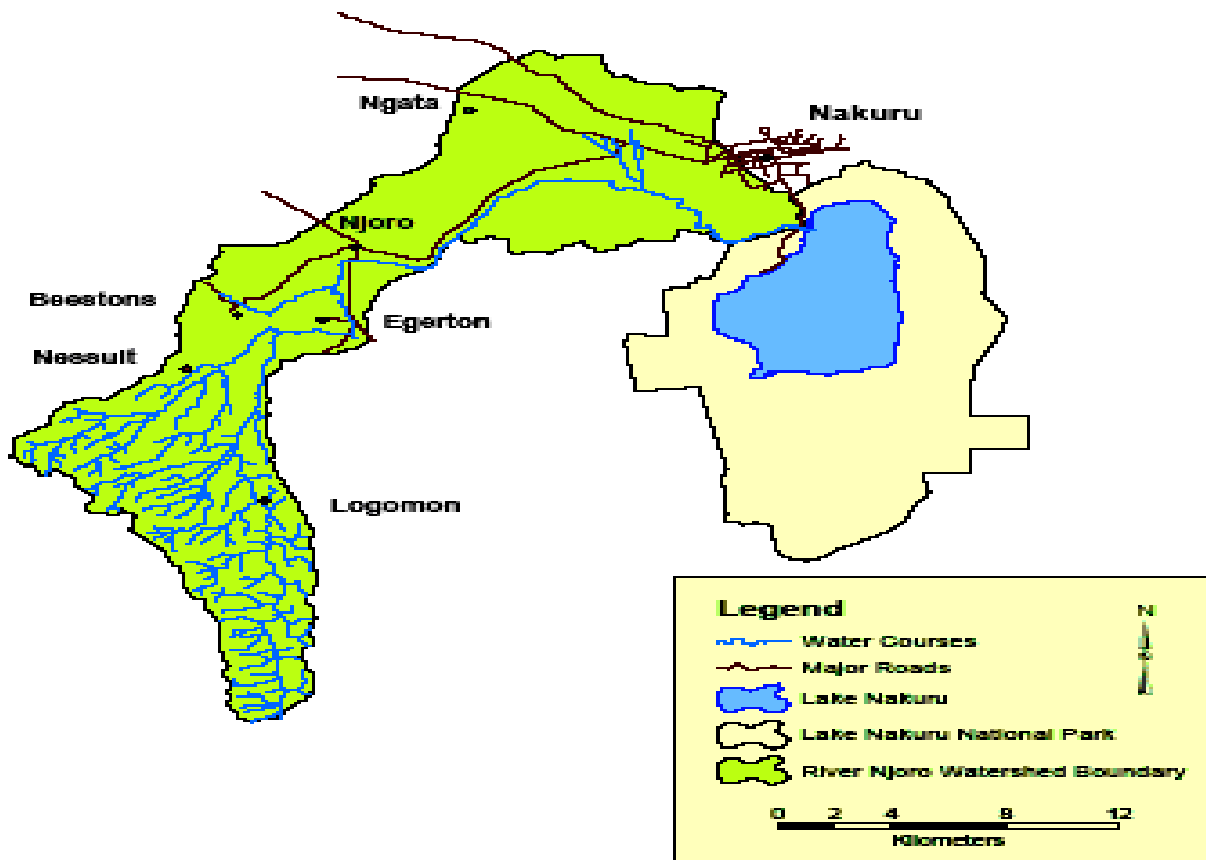

Figure 1. Sampling sites at River Njoro.

Plasmid DNA isolation

The plasmid isolation was done according to the method described by Kado and Liu (1981) with some modifications. Strains were grown in 10 ml of Luria-Bertani (LB) broth containing appropriate antimicrobial agent at 37°C with shaking to exponential stage. Bacteria cells from 1.5 ml of culture were harvested through centrifugation and the pellets were resuspended in 200 µl of Tris-EDTA buffer (40 mM Tris-acetate, 2 mM EDTA [pH 7.9]). The cells were lysed by the addition of 400 µl of lysis solution (containing 3 g Sodium dodecyl sulphate, 0.6 of Tris and 6.4 ml of 2 N NaOH in 100 ml of distilled water, [pH 12.6]). The mixture was incubated at 60°C for 1 h. Protein and chromosomal DNA were then precipitated by the addition of 900 µl of phenol-chloroform 1:1 (v/v) and the precipitate was removed by centrifugation. The supernatant (containing plasmid DNA) was then freed of phenol by extraction with I volume of chloroform. The upper aqueous layer containing the plasmid DNA was collected and subjected to agarose gel electrophoresis for the detection and sizing of plasmid DNA. The gel was prepared at 0.8% in 1X Tris-EDTA buffer and electrophoresis was carried out at 70 V for 2.5 h. Sizes of the plasmids were compared using lambda DNA Hind III Digest, with sizes ranging from 0.56 kb to 23.13 kb.

RESULTS

During sample collection it was assumed that the sampling sites were heavily contaminated. This was confirmed by the evidence of animal faecal contaminants at the sites that would have allowed the growth of bacterial pathogens. In total, 36.5% (46/126) of the samples colected were positive for *A. hydrophila*. The down-stream river section sample sites were heavily contaminated. This is because they pass directly through several built up domestic areas including KARI, Ngata, Njokerio village and Egerton University, where faecal contamination from human and livestock and industrial effluents are discharged into the river. Industrial returned water often has a reduced oxygen level and can result in thermal pollution. KARI may be a possible site for fertili-ser run off into the river, resulting in local eutrophication of River Njoro.

Antimicrobial resistance and plasmid profiles

About 65% (82/126) of the samples were resistant to two or more antimicrobial agents tested (Table 1). Chloramphenicol, nalidixic acid, ciprofloxacin, cefazidime and cefixime were the most sensitive drugs with 100% efficacy whereas ampicillin, cefaclor and streptomycin were the most resistant drugs having 100.0, 67.0 and 50% resistance respectively. There was low resistance against

Table 1. Antimicrobial resistance and plasmid profiles of *A. hydrophila*.

Antimicrobial resistance profiles	Number of Isolates showing similar antimicrobial resistance profile	Mass of plasmid (Kb)	Number of isolates with plasmid
AMP, CEC, ZOX, CN, S	5	21.0	3
AMP, ZOX, S	2	0	0
AMP, CEC, ZOX	1	21.0	1
CEC, ZOX, CN	2	21.0	1
AMP, ZOX	2	-	0
AMP, ZOX, CN, S	5	21.0	5
CN, ZOX, S	3	21.0	1
AMP, S	10	21.0	8

AMP, Ampicillin; CEC, cefaclor; ZOX, ceftizoxime; CFM, cefixime; CAZ, cefazidime; CN, gentamicin; S, streptomycin; C, chloramphenicol; NA, nalidixic acid; CIP, ciprofloxacin.

ceftizoxime (16.7%) and gentamicin (23.3%). These results indicate that all *A. hydrophila* isolated from River Njoro had complete resistance to ampicillin and showed variable resistance to cefaclor, streptomycin, gentamycin and ceftizoxime. Studies revealed that 63.3% of the *A. hydrophila* harboured plasmid of 21.0 kb (Figure 2). Plasmid profiling revealed that most of the multi-drug resistant isolates contained one plasmid of 21.0 kb. Although some strains exhibited different antimicrobial resistance patterns, all of their plasmid was of the same size (21.0 kb).

DISCUSSION

The recent changes in environmental factors combined with the ability of *A. hydrophila* to exist in a diverse set of environments may have allowed *A. hydrophila* to adapt to and occupy a previous non-existing ecological niche. The additional problem of the pollution of the Njoro River may have contributed to the presence of pathogenic strains of *A. hydrophila.* Faecal contamination of the river from both animal and human sources may have led to the hyper-eutrophication of water (Yillia et al., 2008). This factor combined with the increasingly brackish, slow moving tributaries and increased anthropogenic activities might have adversely affected the ecological balance in River Njoro (Mokaya et al., 2004; Yillia et al., 2009). The combination of the above could in essence have created the ecological niche necessary for the proliferation of *A. hydrophila*.

This study indicates that *A. hydrophila* isolated from River Njoro were 100% susceptible to ciprofloxacin, nalidixic acid, cefazidime, cefixime and chloramphenicol. Moreover, the isolates were found to be resistant to gentamicin, streptomycin, ampicillin, cefaclor and ceftizoxime. *A. hydrophila* isolates were all 100% resistant to ampicillin, whereas third-generation cephalosporins such as cefazidime and cefixime and fluoroquinolones (ciprofloxacin and nalidixic acid) were 100% effective.

These results are in accordance with those reported by other authors, showing that third-generation cephalosporins are active against *A. hydrophila* (Poirier et al., 1993). Studies carried out on Aeromonas spp isolated from traveler's diarrhoea patients, demonstrated that the percentage of strains with resistance to chloramphenicol, tetracycline or trimethoprim-sulfamethoxazole ranged from 22.9 to 45.0% (Chang et al., 2007). These levels of resistance to common drugs may be related to the extensive use of these antimicrobial agents in developing countries (CDC, 2003). Elevated levels of resistance against streptomycin have also been detected in both clinical and environmental isolates (Chang et al., 2007). Studies by Chang et al. (2007) and Son et al. (1997) found antimicrobial resistance against ampicillin and streptomycin to be increasing at alarming rates.

Little research has been done previously to evaluate the antimicrobial resistance profiles of *A. hydrophila* isolates from drinking water. Many of the bacteria used in past studies to characterize antimicrobial resistance were clinical isolates (Ko et al., 1996; CDC, 2003) and were rarely Aeromonads (Kiruki et al., 2006). The *A. hydrophila* isolated from River Njoro showed equivalent or much lower resistance compared to *A. hydrophila* isolates reported in other studies depending upon the antimicrobial agents tested. A possible explanation for these findings is that some previous studies used clinical isolates that could have been previously exposed to antimicrobial agents (Poirier et al., 1993; Vila et al., 2003).

Presence of plasmid DNA in *A. hydrophila* multi-drug resistance strains demonstrated that resistance was plasmid mediated and this could have resulted from cross-transmission of resistant isolates between people and consumption of food from animals that have received antimicrobial agents. Frequent use of antimicrobial agents in medicine and in food of animal origin production has resulted in an increase in the prevalence of bacterial strains resistant to these antimicrobial agents (Ko et al., 1998; Power, 2006; Olusesan et al., 2010).

In Kenya, there is an increase in use of antimicrobial

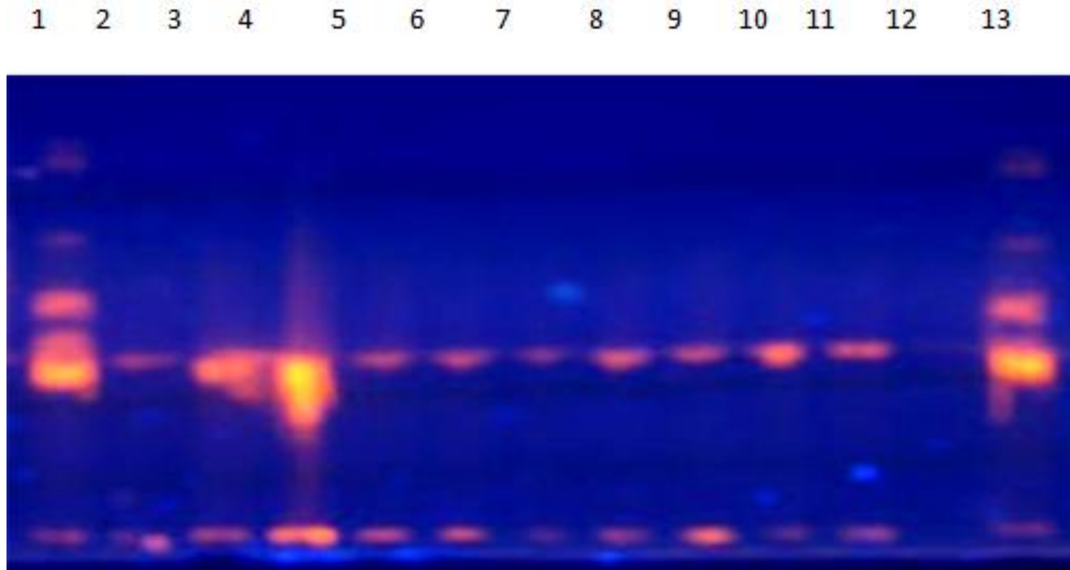

Figure 2. Ethidium bromide-stained agarose gel (0.8%) containing plasmid DNA of the indicated strains. Lanes 1 and 13, having marker Lambda DNA; Hind III digest (Invitrogen Corp. USA); lane 2, A positive control of *A. hydrophila*; lanes 3 and 4, *E. coli* strains (with plasmid of 23.13 kb); lanes 5 to 11, *A . hydrophila*; lane 12, negative control lane.

agents as food additives to improve growth and feed conversion in many types of animals resulting in accumulation of these drugs among bacterial communities in the intestinal tracts of domestic animals (Mitema et al., 2001; Levy, 2002; Power, 2006). This work revealed that the use of effective drugs against natural strains will cause them to disappear and then be replaced later by the drug resistant strains. The new strains will be responsible for potentially severe infections in the community (Carbon and Isturiz, 2002; Nova'kova et al., 2009). It has been reported that the occurrence of plasmid bearing one or more resistance genes such as R-plasmid observed in this study possesses the ability to often code for enzymes that destroy drugs, or alter the affected pathway (Walsh et al., 1995; Salyers, 2002; Kummerer, 2004). According to Olusesan et al. (2010), once a bacterial cell possesses an R-plasmid, the plasmid may be transferred to other cells quite rapidly through normal gene exchange processes such as conjugation, transduction and transformation. This gene exchange may lead to a single plasmid carrying gene for resistance to several antimicrobial agents. Through the acquisition of plasmid conferring multi-drug resistance, the strain undergoes the necessary and appropriate adaptation for survival in the changing antimicrobial environment.

The isolation of *A. hydrophila* with only one plasmid (21.0 kb) suggests that other R-plasmids might have been lost in the environment or their antimicrobial resistance is chromosomal, transposons or intergron mediated. The study also indicates that plasmid 21.0 kb is common in *A. hydrophila* and is important for antimicrobial resistance and virulence.

The reason for the high resistance to common drugs observed in this study may be due to their low cost, thus increasing an irrational consumption rate, transmission of resistant isolates between people and consumption of food from animals that have received antimicrobial treatment. The problem of antimicrobial resistance in bacteria enteropathogens typifies the growing concern among health care workers on the continued effectiveness of antimicrobial empiric management of infections. At this juncture, it is critical to recall that the essence of monitoring antimicrobial resistance profiles among enteric pathogens is to provide updated data for clinicians in order to facilitate the use of appropriate and more effective treatment regimens. Self medication and non-compliance with medication and sales of substandard drugs may account for the rise in antimicrobial resistance observed in this region. The greater the overuse of antimicrobial agents, the more the elimination of the sensitive strains allowing resistant strains to dominate. It is well established that antimicrobial pressure supports resistant strains and eliminates sensitive strains. It is also true that resistant strains are outcompeted by sensitive strains when antimicrobial pressure is removed from the environment (CDC, 2003). Thus, steps must be taken to control the overuse of antimicrobial agents in Njoro region.

Conclusion

This study confirmed the role of River Njoro as a reservoir of antibiotic resistance *A. hydrophila* that contained 21.0 kb plasmid, which can disseminate antibiotic resistance genes to other human pathogens and so constitute

a problem for human health.

Therefore, it will be vital for public health workers to create awareness for the need to observe good health practices, boil drinking water and seek alternative sources of drinking water in the study area. The medical personnel should investigate and ascertain the prevalence of *A. hydrophila* in both clinical and water samples. The problem posed by antibiotic resistance among enteropathogenic bacteria isolated from River Njoro necessitates the need to ascertain any association or linkage between enteric pathogens from clinical samples such as stool and River Njoro water which may be common source of diarrhoea infections in the area. Further studies are required to ascertain the role of 21.0 kb plasmid as a virulence marker in A. hydrophila.

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