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Full Length Research Paper

Isolation of *Legionella pneumophila* from surface and ground waters in Osogbo, Nigeria

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Legionella **is a ubiquitous water environmental organism. Isolation/detection of Legionellae has been reported worldwide. However, there is no reported isolation of Legionellae in countries in Africa with the exception of South Africa. The aim of this study was to survey the surface, ground waters and air conditioner water systems (ACWs) in Osogbo, Nigeria for the presence of** ***Legionella pneumophila*** **by cultural isolation method. A total of 313 water samples from the streams (surface water), wells (groundwater) and ACWS were cultured on buffered charcoal yeast extract (BCYE) medium. The isolates were identified by Gram staining techniques, biochemical tests and demonstration of pore forming activity. Polymerase chain reaction for amplification of 0.52 kb fragment of the part of dot/icm region of** ***L. pneumophila*** **was used for the final identification. The results obtained showed that the prevalence of** ***L. pneumophila*** **from streams (8%) was statistically significant when compared to wells (1%) (Chi square, P < 0.05).** ***L. pneumophila*** **was not isolated from any of the water samples taken from air-conditioner water systems. The study concludes that** ***L. pneumophila*** **is present in natural aquatic environment in Nigeria.**

Key words: Cultural isolation, *Legionella pneumophila*, Nigeria.

INTRODUCTION

Legionella sp is an environmental organism that has gained recognition as a public health organism over the years as a result of changes in our life style. It is a Gram negative, intracellular pathogen that has the ability to survive within the amoebae and macrophages (Abu Kwaik et al., 1998; Adeleke et al., 1996; Alli et al., 2002). The organism was first brought to a limelight in 1976 as a result of Legionnaire convention in Philadelphia, United States of America (Fraser et al., 1977). From its natural environment - water, legionellae can be transmitted to humans by inhalation of infectious droplets. There are at least 48 species of *Legionella* that have been identified of which five species have been designated *Legionella like* amoebic pathogens (LLAPs) (Adeleke et al., 1996; Lo Presti et al., 2001) and there is still room for discovery of new species as a result of more surveillance of water bodies. Within the species of *Legionella* are 70 serogroups with more than half been implicated in diseases (Benson and Fields, 1998; Fields et al., 2002). *Legionella pneumophila* is responsible for most cases of Legionnaires' disease in the world, with *L. micdadei* coming distantly second (Benin et al., 2002; Joshi and Swanson, 1999). *L. longbeachae* and *L. dumoffii* rank third and fourth, respectively (Benin et al., 2002).

Legionellae multiply within its host cell (human macrophages and amoebae) but can also grow outside its host cell and can be cultured by routine methodologies that involve the use of selective medium (Feeley et al., 1978; 1979). In aquatic environments, *L. pneumophila* replicates within protozoan hosts. At least 13 species of amoebae and 2 species of ciliated protozoa support the intracellular growth of *L. pneumophila* (Fields, 1996). The dot/icm loci have been shown to play important roles in the pathogenicity of Legionellae (Berger and Isberg, 1993; Marra et al., 1992; Molmeret et al., 2002a; 2002b). In fact, the loci have been demonstrated to help in the

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intracellular multiplication in both protozoan and macrophages (Brand et al., 1994; Segal and Shuman, 1999).

After intracellular multipli-cation and exhaustion of nutrients in the hosts, in which the host cells are filled with the bacteria, Legionellae use the dot/icm to egress from the host cells as a result of pore forming activity of the bacteria that help in escaping the cell so as to infect fresh host cells (Alli et al., 2002; Molmeret et al., 2002a; 2002b). Inside the macrophage, the phagosome is prevented from fusing with lysosome, hence the bacteria live happily inside the cells (Horwitz and Maxfield, 1984). Apoptosis and necrosis have been shown to contribute to cell death of the host cells (Gao and Abu Kwaik, 1999; 2000). The dot/icm loci have been demonstrated in all species of Legionellae to date by hybridisation (Alli et al., 2003), however, the functionality of these loci are in doubt.

Epidemiologically, the first outbreak of legionellosis was reported in 1976 in Philadephia in USA, an outbreak that claimed the lives of many attendees of Legionnaires' convention (Fraser et al., 1977). Since then, outbreaks of legionellosis had been reported all over the world. In USA where surveillance for legionellosis is put in place, between 1980 and 1998, an average of 356 cases were reported to CDC each year with no particular trend (Fields et al., 2002). This is considered to be a fraction of 8,000 to 18,000 cases estimated to occur each year (Marston et al., 1997). Majority of the cases reported in the USA are sporadic. Outbreaks in the USA have been frequently associated with cooling towers in summer and fall (Bentham and Broadbent, 1993). In England and Wales, the Legionellae surveillance carried out showed a similar trend to what had been reported in the USA, reaching its peak in 1988 (Joseph et al., 1995; 1997).

In Holland, the 1999 outbreak of *Legionella* infection among attendees at flower show resulted in 133 confirmed and 55 probable cases of Legionellae (Den Boer et al., 2002). April 2000 outbreak of legionellosis in Australia among people visiting newly constructed aquarium in Melbourne claimed 4 lives with total number of people infected put at 119 (Anonymous, 2000). In Africa, the only country that reported outbreak of *Legionella* in a hospital is South Africa (Strebel et al., 1988).

Apart from this, many researchers all over the world including South Africa have reported the detection and isolation of Legionellae in their aquatic environments (Bartie et al., 2003; Erdogan and Arslan, 2007; Kuroki et al., 2009). It is inconceivable to think that *L. pneumophila* is not present in the authors' aquatic environment. The only information we have on the possible presence of Legionellae can be derived from seroprevalence study conducted about two decades where they reported 9% seroprevalence to *Legionella* (Sixl et al., 1987), suggesting possible exposure to Legionellae. Since the seroprevalence study, no researcher has reported the detection/isolation of Legionellae in water bodies in Nigeria or West Africa. The information on this is needed to determine the risk of acquiring Legionellae from the environmental irrespective of whether it is man-made or natural. It was in view of this that this study was instituted. The aim of this study was to survey aquatic environments for the presence of *L. pneumophila* in order to determine the prevalence of this species of *Legionella* in water bodies and air conditioner water system in Osogbo, Nigeria.

MATERIALS AND METHODS

Sample collection

Three hundred and thirteen (313) water samples from three different sources (air conditioner water systems, wells and streams) were examined for the presence of *Legionella* spp, of which 165 samples were collected from air conditioners while 98 and 50 samples were collected from wells and streams, respectively. Samples for investigation were collected from different locations within Osogbo, Osun State, Nigeria. One hundred millilitre volumes were collected from wells and streams into a sterile wide mouth screw capped specimen bottles, respectively. Swab sticks were used to scrape the mouth of air conditioner pipes after which 100 ml of water draining from the pipe was collected. All samples were stored in the laboratory at room temperature before investigation commenced.

The sample was processed for isolation of *Legionella* spp. as described in standard microbiology manual (Collins et al., 2004). A volume of 100 ml of each sample was placed in centrifuge tube and centrifuged at 5000 x g for 10 min, after which the supernatant was discarded carefully leaving about 2 ml of water with the sediment. The sediment was vortex mixed for one minute. Thereafter, 1 ml of sample concentrate was incubated at 50°C in a water bath for 30 min; in order to reduce the number of non-*Legionella* bacteria from water samples before culture, and the remaining samples were stored at room temperature for possible use later. The heated samples were placed on bench for 10 min to attain room temperature.

Cultural isolation and Identification

About 100 µl of the treated sample suspension above was inoculated onto buffered charcoal yeast extract (BCYE) medium. After inoculation, the plate was incubated at 37°C in a humidified 5% CO_2 incubator. All cultures were examined after 48 h for possible bacteria growth and plates which showed no growth was re-incubated for another 24 h. Macroscopic examination of culture was thereafter done to identify typical Legionellae colonies. Each suspected colony was aseptically sub-cultured on BCYE agar containing supplement (cysteine and ferric chloride) and BCYE agar with no supplement. Isolates that were able to grow on BCYE agar with supplement and not able to grow on BCYE without supplement were taken for further identification as described below. Only isolates that were Gram negative rods were selected for biochemical test. Catalase, oxidase, hippurate hydrolysis and gelatine liquefaction tests were carried out as described in a standard medical microbiology laboratory manual (Cheesbrough, 2004).

Pore forming activity

Pore forming activity was determined by examining haemolysis of

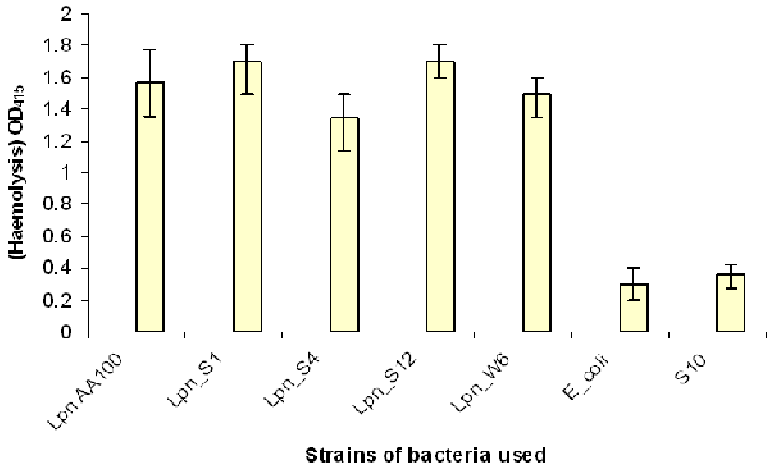

Figure 1. Pore-forming activity of Legionella spp. as determined by contact-dependent haemolysis of sRBC measured as A415. These data are representative of at least three independent experiments performed in triplicate. The absence of error bars indicates very small standard deviations that could not be displayed.

sheep red blood cells (sRBC) by suspected Legionellae isolates as previously described (Alli et al., 2003). Briefly, sheep red blood cells (sRBC) were washed three times by centrifugation for 10 min at 500 x g until a clear supernatant was seen; the cells were then counted with a haemocytometer. Reactions were set up in a final volume of 1 ml PBS with final concentrations of 1 x 10^8 sRBC ml^{-1} and 2.5 x 10^9 bacterial ml^{-1} and incubated at 37°C for 2 h. At the end of incubation period, centrifugation was done at 500 x g for 5 min. Tubes were then observed for haemolysis. Negative control for haemolytic assay was set up using *E. coli.*

Polymerase Chain Reaction (PCR)

PCR for part of the *dot/icm* region was carried out on all suspected isolates that were oxidase, hippurate hydrolysis, and gelatine liquefaction positives. Briefly, the culture lysate obtained by boiling colonies of suspected organism in 200 µl of distilled water in a 1.5 ml Eppendorf tube for 10 min and chilled was used. The 522 bp of the *dot/icm* region was amplified in a 30 µl PCR reaction volume containing $MgCl_2$, Taq DNA polymerase (New England Biolab, USA) , 1 µM of Lpn_dot_F1 (GCAATCTTCAGTCCTGGGAG) and 1 µM of Lpn_dot_B1 (TGCTGCTCTTGTTGTGCCA) in a Gene Amp PCR system 9700 (Applied Biosystem, UK) using the following cycling parameters: 94°C for 2 min 1 cycle; 94°C for 30 s, 56°C for 30 s, 72°C for 1 min, for 35 cycles. The positive control was included by using the DNA obtained from *L. pneumophila* AA100. Thereafter, the PCR products were analysed on 0.7% agarose gel in Tris Borate EDTA buffer solution, the image was taken with Syngene gel documentation system (Syngene, UK).

Statistical analysis

Chi square was calculated using EpiInfo version 6 where P value less than 0.05 was considered to be significant.

RESULTS

In a survey of the environment containing water (both man made and natural water bodies) in Osogbo, Nigeria, for *L. pneumophila*, a total of 313 samples was collected, of which 165 (52.7%) were from air conditioner water systems, 98 (31.3%) were from streams and 50 (16%) were from the wells.

Out of the 313 water samples cultured, 179 (57.2%) samples yielded growth while 134 (42.8%) samples yielded no growth. From air conditioner, 81 (49%) samples yielded growth while 84 (51%) samples yielded no growth; from well water 66 (67.3%) samples yielded growth while 32 (32.7%) yielded no growth; and from stream 32 (64%) samples yielded growth while 18 (36%) samples yielded no growth. The suspected bacterial colonies were put through systematic identification of Legionellae by sub-culturing on both BCYE agar medium without supplement (cysteine and ferric chloride) and BCYE agar medium with supplement including antibiotics. This strategy pruned down the number of bacterial isolates down to 5, of which all of them were Gram negative rods exhibiting both catalase and oxidase activities with hippurate hydrolysis and gelatine liquefaction – a biochemical result suggestive of *Legionella* spp. Alli et al. (2003) have shown before that pore forming activity within the genus *Legionella* is associated with *L. pneumophila* and *L. spirintensis*. It was in view of this that pore forming activity was tested for in all the 5 suspected legionellae isolates by performing haemolysis assay in which sheep red blood cells were used, the result (Figure 1) showed that all exhibited pore forming activity that was very close to that of *L. pneumophila* AA100 that was used as a positive control. Further identification was carried out at molecular level by doing polymerase chain reaction for specific detection of 0.52 kb fragment of the dot/icm region of *L. pneumophila*; this revealed that all the five isolates had the 0.52 kb

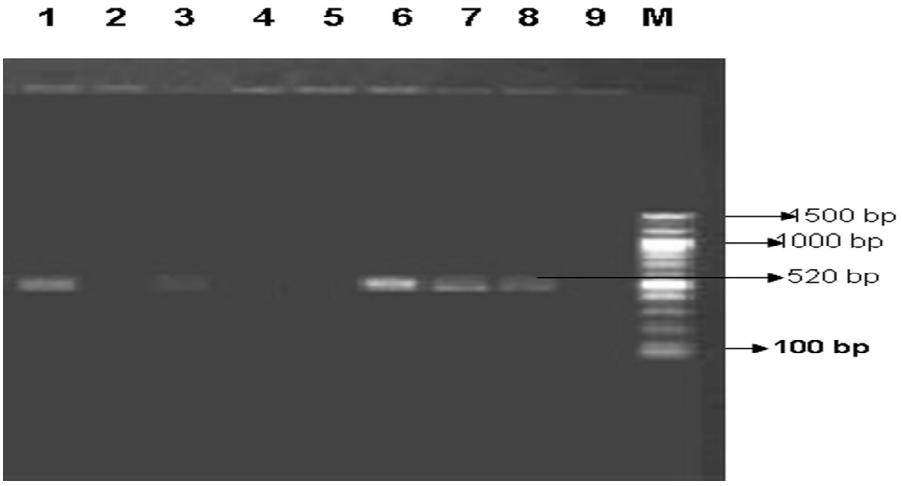

Figure 2. PCR for detection of 0.52 kb region of the dot/icm of *L. pneumophila*. The PCR products obtained after PCR were run on 0.7% agarose gel as described in the materials and methods. Lanes 1-7: PCR for the colony lysates from suspected legionellae; lane 8: PCR product for AA100 *L. pneumophila* DNA as positive control; lane 9: PCR product for water as negative control and lane 10: 100 bp DNA size marker.

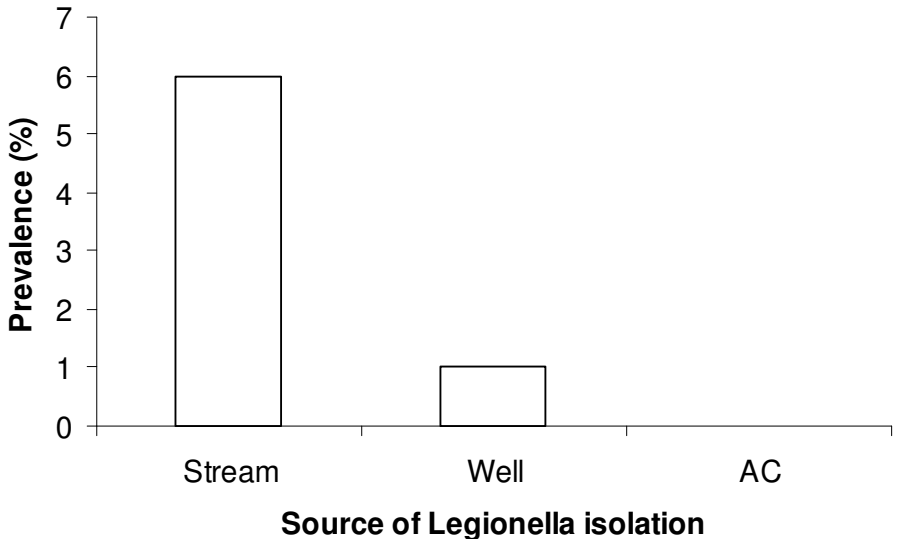

Figure 3. The prevalence of *L. pneumophila* in different sources of water in Osogbo, Nigeria.

fragment of the *dot/icm* region of *L. pneumophila* as shown in Figure 2, indicating that all the 5 isolates (Lpn_S1, Lpn_S4, Lpn_S12, and Lpn_W6) were *L. pneumophila*. The prevalence of *L. pneumophila* in stream samples was calculated to be 8% (4/50) while the prevalence in water from the well was found to be 1.02% (1/98) (Figure 3). There was significant difference in the rate of isolation of *L. pneumophila* between stream and well water samples (Chi square, $P < 0.05$). Surprisingly, *L. pneumophila* was not isolated in any of the air conditioner water system samples.

DISCUSSION

Legionellae are readily found in natural aquatic bodies with some species been recovered from soil (Steele et al., 1990). Studies have shown that Legionellae are present in all segments of community water supplies, water treatment facilities (Erdogan and Arslan, 2007; Kuroki et al., 2009). The aquatic environment of *Legionella* also includes man made habitats such as cooling towers, evaporative condensers, whirlpool spas, decorative fountains, air conditioner water systems, and

potable-water distribution systems. Our study showed that Nigeria is not exempted from the list of countries where *Legionella* are found. Moreover, it also showed that the most virulent species of *Legionella* (*L. pneumophila*) is present. *L. pneumophila* was shown to be prevalent (3.4%) in natural aquatic habitats ground-water (well) and surface water (stream) compared to and man-made (water of condensation from air conditioner water system) in this study.

No reported isolation or detection of Legionellae in countries in Africa with the exception of South Africa. The only evidence available suggesting that Legionellae may be present in Nigeria was the seroprevalence study carried out by Sixl et al. (1987) where the seroprevalence to *L. pneumophila* was put at 9% among the blood donors in Abeokuta and Minna (Sixl et al., 1987). Recently, Olukanni et al. (2006) reported the isolation of *Legionella* in textile effluent using nutrient agar (Olukanni et al., 2006) and this is doubtful because legionellae require special medium to grow. Our study showed for the first time the reported isolation of *L. pneumophila* in aquatic environments (surface water and groundwater) with full identification of the isolates by both biochemical and molecular methods. It means these environments pose a risk in contracting Legionellae especially to immunocompromised individuals. The use of selective media like BCYE agar medium has proven to be very important in the isolation of Legionellae in this study as demonstrated by other researchers in this field (Azara et al., 2006; Bartie et al., 2003). Legionellae have been shown to be very exact in its nutritional demand and this might be the main reason for the non reported isolation of Legionellae in Nigeria and some other countries in Africa. It is also not a pathogen that is routinely checked in respiratory tract infection. The only country that reported the outbreak of Legionellae in Africa including the environmental isolation is South Africa. The general awareness of this organism in their environment may be responsible for the reported isolation of Legionellae in the hospital settings including dental clinics and the communities outside the hospital. Our experience in the use of BCYE agar medium also showed that the medium alone was not sufficient for the selective isolation of Legionellae because environmental organisms seemed to come through despite the incorporation of antibiotics that selectively inhibit the growth of competing microflora of the environments including fungi. This study is in line with other studies where the BCYE with antibiotics was used in the detection and isolation of Legionellae from the environments (Lin et al., 1999; Stout et al., 2003).

Identification of Legionellae could be problematic because these organisms have limited metabolic activities when compared to *Enterobacteriacea* and other non-fastidious organisms. This study made use of a simple schema where it exploited the inability of the organism to grow in the absence of cysteine cum iron and the ability to grow in the presence of cysteine and iron as the screening point. This alone narrowed the number of initial isolates to manageable number of isolates that were systematically identified using Gram stain, biochemical tests such as gelatine liquefaction and hippurate hyrolysis. These biochemical tests combined with demonstration of pore forming activity of *L. pneumophila* helped in narrowing down to *L. pneumophila*. Pore forming activity has been described as one of the virulence traits of *L. pneumophila* (Alli et al., 2003) which is best demonstrated by haemolysis assay using sheep red blood cells. Combining this haemolysis assay and the ability to amplify the 0.52 kb portion of the dot/icm region of *L. pneumophila* helped in the final identification. The demonstration of the 0.52 kb region of the dot/icm also indicates that the pathogenic island is present in Nigeria isolates of *L. pneumophila*, which means the organism is capable of causing infection and disease if the organism is inhaled in large number by individuals.

In this study, isolates from stream water showed the highest recovery of *L. pneumophila* as shown in Figure 3, followed by well water isolate. This high prevalence in stream can be attributed to the interaction of *L. pneumophila* with free-living amoeba which facilitates multiplication of the bacteria in their natural environ-ment. Although no air conditioner water sample yielded growth of Legionellae, compared to only one that was positive for well; well was regarded as the 2nd implicating reservoir of *Legionella*. The non isolation of Legionellae from air conditioner water systems can be attributed to being an artificial man-made that is devoid of amoeba that is supportive of Legionellae growth unlike stream and well that are natural habitats for Legionellae and amoebae. Air conditioner water system has been implicated as a high yield recovery of *Legionella.* In a survey carried out on the detection of *Legionella* in Germany, 15 water samples from cooling towers of air-conditioning systems at hospitals, authorities, schools, and factories were collected and *Legionella* was isolated from about 2 of the air-conditioners investigated on in that study area, thus giving a prevalence of 3.3% (Dermitzel et al., 1992). In our survey, none of the 165 water samples from air-conditioning systems was positive for *L. pneumophila* giving a prevalence of 0%. Thus the prevalence in Germany is higher than that of ours. A similar study carried out in Iran, 30 out of 132 (22.72%) hospital water samples collected from different sources were positive for *L. pneumophila* using PCR technique of which air conditioner water sample gave a high yield positivity of 10 out of the 30 positives (Hosseini et al., 2008). The study showed air conditioner as being the most implicated source of Legionellae, in contrast to our study where air conditioner was not implicated as a source of *L. pneumophila* infection. The inability to isolate *L. pneumophila* from air conditioning systems in this study could be attributed to undulating and epileptic power supply in Nigeria. When power failure occurs, air

conditioning systems stops functioning, air conditioning pipes/outlets get dried up, and *Legionella* yield within pipe/outlets is greatly reduced because a lot of the bacteria would have died. Notwithstanding, *L. pneumophila* was detected in both streams (surface water) and wells (ground water). This study is comparable to a study conducted in Netherlands where PCR technique was used for detection of legionellae in groundwater (well) and surface water (stream); surface water showed a high yield of *Legionella* organism compared to groundwater water samples (Wullings and van der Kooij, 2006).

In this study, the total prevalence of *L. pneumophila* from environmental water bodies (streams and wells) in Osogbo, Nigeria was calculated to be 3.4% in relation to 148 water samples collected. Its detection is an indication of the presence of *Legionella* in Nigeria. Although the prevalence might be small, its presence leaves Nigerians at risk of contracting the disease, in that people could get infected with the disease from areas where streams and wells are present. Water samples from air-conditioning system obtained from the hospital environment were implicated in being contami-nated with *L. pneumophila*, the most pathogenic strain and it has been shown that cooling towers of air-conditioning systems have been implicated for nosocomial legionellosis (Dermitzel et al., 1992). Recent studies, documenting the genetic identity of clinical and environmental isolates have proven that local outbreaks of legionnaire`s disease may indeed originate from contaminated cooling towers (Hosseini et al., 2008). *Legionella* residents within biofilm are a particular problem in cooling tower systems. *Legionella* was also detected in well water which serves as a means of water supply in Nigeria. Well water is fetched directly for use in bathing, washing, and for other domestic chores. It is also pumped directly, and run through pipes to store water into overhead tanks to supply to man-made fountains, which generates aerosols. In some rural areas and villages, water from the streams is used for bathing, and cooking. These practices make Nigerians to be at a higher risk in contracting aerosols contaminated with the bacteria. The study concludes that *L. pneumophila* is present in water bodies in Nigeria. Translation of the presence of Legionellae in water bodies to risk of contracting Legionellae infection is remained to be seen.

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