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Interaction of Legionellae and Free-Living Amoebae Within Hospital Water Supplies

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Abstract: The aim of current study was to investigate the interaction of free-living amoebae and Legionellae in Iranian hospital water supplies. Contamination of hospitals water supplies with Legionellae is a well-known cause of nosocomial Legionnaire's disease. Free-Living Amoebae are known to harbor a range of opportunistic microbial pathogens such as Legionellae, sequestering them from antimicrobial agents as well as environmental stresses. Less is known however of the interaction between the thermotolerant free-living amoebae and Legionellae. A total of 115 water samples from medical institutions across the nation were screened for Legionellae and Free-Living Amoebae co-existence by culture and PCR method. Electron microscopy (TEM) method were used to investigate intra-amoeba multiplication of legionella. The sampling sources were selected randomly among the major hospitals located in 13 province centers. *L. pneumophila* were isolated from 39% of water samples by PCR and culture methods. Different species of Free-Living Amoeba including *Ackanthamoeba* sp. were isolated from 46% of samples. Thirty percent of amoebae positive samples were also positive for Legionellae. Fifty one percent of Legionellae isolates were identified as Lp 7-14 and 49% were non *L. pneumophila* species. Electron microscopy revealed intracellular proliferation of bacteria. The residual chlorine concentration of 63% of tap water samples was 0.2-0.4 mg mL^{-1} while their pH was between 6-7. More than half of the water samples belonged to major hospitals in the region. This problem, however, not limited to hospitals. Most buildings, in Iran use humidified ventilators which are supplied with tap water. The results address the potential threat to the health of public in general and hospitalized patients in particular.

Key words: Legionellosis, water supplies, nosocomial, Iran

INTRODUCTION

The species within the genus *Legionella* were described since the first outbreak of leginnaires' disease (Fields *et al*., 2002). Since the first description of the genus, 48 species and more than 69 serogroup of legionella have been identified. Twenty species with 39 serotype have been related to human disease (Fields *et al*., 2002). *Legionella* have been recognized as the etiological agent of legionnaires' disease and Pontiac fever. *Legionella* species are ubiquitous in environmental waters and capable of surviving extreme ranges of environmental conditions (Bouyer *et al*., 2007). The interaction with other micro organism seems to increase the resistance of *Legionellae* to thermal treatment and also some other biocides including free chlorine (Storey *et al*., 2004).

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Human infection occurs through the inhalation of aerosols contaminated with *Legionella* species (Pankhurst and Coulter, 2007). Potential sources of legionellosis include *Legionella*-contaminated water in cooling towers and air conditioners, hot tubes, showerhead water and public fountains (Barbaree *et al*., 1993). More recent investigations have emphasized the role of either aspiration or ingestion of infective water as important modes of acquiring disease (Stout *et al*., 1992). The contamination of environmental waters with *Legionellae* has been reported from almost globally (Fields *et al*., 2002; Singh and Coogan, 2005; Armstrong and Haas, 2008).

Free-Living Amoebae (FLA) are ubiquitous organisms that have been isolated from various globally water sources, such as cooling towers and hospital water networks (Thomas *et al*., 2006). The cyst forms of some FLA remained viable after treatment with up to 100 mg L^{-1} chlorine for 10 min, as well as 80°C heat (Storey *et al*., 2004). In addition to their own pathogenicity, FLA can also act as Trojan horses and be naturally infected with amoebae-resisting bacteria that may be involved in human infections (Thomas *et al*., 2006). Since sporadic nosocomial and community acquired legionellosis have been reported from Iran (Doust *et al*., 2008) and some of the regional countries (Ozerol *et al*., 2005), the interaction between *Legionellae* and Free-living Amoebae in Iranian hospital water supplies were investigated in present study.

MATERIALS AND METHODS

Study Design

From May to August 2007, 115 samples were collected from the water network of the University Hospital in Tehran and other provincial cities of Iran. A total of 69 tap water samples, 30 water samples of shower head and 16 water samples of ventilators. The origins of the samples included the intensive care unit (46 samples), a surgery ward (30 samples) and an internal medicine ward (20 samples). The 19 remaining samples were collected in other wards of the hospitals.

Temperature and other physical characters were recorded for each water sample at the moment of sampling. Water samples were collected from each hospital point in 4 L plastic container according to methods earlierly described (Hosseini-Doust and seal, 1998). The physical properties and free chlorine residuals of each water sample were recorded before transfer them immediately to central research laboratory for next procedures.

Each water sample was filtered using a peristaltic pump as earlierly described (Hosseini-Doust and Seal, 1998). Briefly, water sample were filtered through multipore nylon membrane filters, pore size 0.22-0.45 μm filtration and stored at room temperature until used. Turbid samples or those containing particulate debris were filtered through a 1.5 μm pre-filter prior to the main filtration step. After filtration of each sample, the filter stand and pipework systems were pasteurized by passing boiling water through assembly. Boiling water passed in this manner for approximately 5 min eradicated any contaminating vegetative organizations. The assembly was then left to cool or cooled by passing of cold sterile distilled water through the system. The filter membrane of each sample were placed in a 50 mL of aliquot of filtrate collected in a sterile boney jar and shaken vigorously to remove any retenate from the membrane before storage at 4°C until required.

Legionella Culture

Buffered Charcoal Yeast Extract (BCYE) agar, supplemented with L-cystein, ferric pyrophosphate and α-ketoglutarate were formulated according standard protocols by some modifications (Hosseini-Doust and Mohebbati-Mobarez, 1999). The BCYE medium was semi-selected with adding Glycine, Cyclohexamide, Vancomycin and Polymixin. The prepared BCYE plates were quality control and stored at +4°C until use. Five milliliter aliquots were removed from each water sample and treated with heating at 56°C for 15 min. The BCYE plates were then inoculated in duplicate with aliquots (0.1 mL) from each water samples. Plates were inoculated for up to 14 days at 37°C

in presence of 5% Carbon deoxide in humid air and growth recorded on the 3rd, 5th, 7th, 10th and 14th day. Direct culturing of the 50 mL aliquots containing the filters was also performed in duplicate as above. Colonies of *Legionellae* were recognized by their appearance and characteristics fluorescence under UV light (Harrison and Taylor, 1988). Colonies were verified as being *Legionella* species by failing to grow on subculture to non-supplemented BCYE agar or fresh blood agar. Presumptive *Legionella* colonies were examined using direct immunofluorescent antibody test according to manufacturer (MAST Media). A commercially available latex agglutination test kit (Oxoid, UK) was used for the final confirmation of *Legionella* colonies. The kit was specific for *L. pneumophila* serogroup 1-14 and seven other non *L. pneumophila*.

FLA Isolation

Peptone-yeast extract-glucose medium contained (in 5 L of distilled water) 100 g proteose peptone (Difco, Sparks, MD), 10 g yeast extract (Difco), 4.9 g $MgSO_4 \cdot 7H_2O$, 5 g sodium citrate $\cdot 2H_2O$, 0.1 g $Fe(NH_4)_2 (SO_4)_2 \cdot 6H_2O$, 1.7 g KH_2PO_4, 1.97 g $Na_2HPO_4 \cdot 7H_2O$, 45 g glucose and 0.295 g $CaCl_2$. Page's modified Neff's amoeba saline (PAS) contained (in 1 L of distilled water) 120 mg NaCl, 4 mg $MgSO_4 \cdot 7H_2O$, 4 mg $CaCl_2 \cdot 2H_2O$, 142 mg Na_2HPO_4 and 136 mg KH_2PO_4. To prepare non-nutritive agar plates, 1.5 g agar (Research Organics, Cleveland, OH) was diluted in 100 mL of PAS (Greub *et al*., 2004). Aliquots of 2 mL of each water samples were mixed with an equal volume of amoeba saline in a 5 mL glass bijou bottle and kept at +4°C (Hosseini-Doust and Mobarez, 2000). Samples (0.1 mL) from these bijous were inoculated onto the surface of non-nutrient agar plates seeded with heat killed *E. coli* to grow free-living amoebae (Page, 1987). The plates were left to air dray and incubated at 25-30°C for up to 15 days, with daily examination by inverted light microscopy after the first week of growth. The positive samples were chosen for subculture onto the fresh non-nutrient plates for axenic culture preparation of FLA (Page, 1967).

PCR for *Legionella*

A thermic-lysis procedure was used to release the bacterial DNA extraction, as follows (Hay *et al*., 1995). A total of 1000 µL of each water sample was boiled for 5 min in a 1.5 mL microcentrifuge tube and chilled on ice for 5 min; this procedure was repeated once. After 2 min of centrifugation at 1100 x g, the supernatant were removed and 10 µL of sediment suspension were used for PCR analysis.

A set of primers were chosen from the *mip* of *L. pneumophila* which was described earlier (Julhac *et al*., 1998). The *mip*, which codes for a virulence protein, seemed to be a promising target for the detection of legionellosis. Two 20-base oligonuclotides (L.pm-1, L.pm-2) bracketing a 600 bp DNA fragment was synthesized as primers. L.pm-1 (5′-GGTGACTGCGGCTGTTATGG-3′) was located at nucleotides 853 to 872 from the coding strand; L.pm-2 (5′-GGCCAATAGGTCCGCCAACG-3′) was located at nucleotides 1465 to 1484 complementary to the coding strand. Samples were submitted to 40 cycles of amplification in a 50 µL volume containing 1 U of Taq polymerase, 0.1 µL each primer, 0.2 mM the four deoxynucleotides (each), 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$. After an initial denaturation step at 95°C for 5 min, each amplification cycle was performed at follows; 2 min for annealing at 62°C, 1.5 min for primer extension at 72°C and 1.5 min for denaturation at 94°C. Ten microliter of each sample containing amplified DNA was electrophorsed through a 1.5% agarose gel in 0.5x TEB buffer (44.5 mM Tris-HCl, 44.5 mM boric acid, 1.25 mM disodium EDTA (pH 8.3)), after staining with ethidium bromide photographed under UV light. For RFLP analysis, 10-20 µL of PCR products were digested with kpn1 restriction enzyme (Fermentas) according to manufacturer's and the restriction fragments were analyzed by gel electrophoresis at 120 V and stained with ethidium bromide. The PCR products were confirmed using kpn1 restriction enzyme which cuts the 630 kbp product into 400 and 230 kbp specific fragments.

Electron Microscopy

The amoeba-*Legionella* electron microscopy procedures were achieved by methods previously described (Lewis and Knight, 1977). Briefly, the FLA contaminated with *Legionellae* grown as axenic culture in tissue culture flask. The amoebae cells were harvested and washed three time with pre-warmed amoeba saline to remove the extracellular bacteria. Amoebae were then fixed in 0.1 M cacodilate buffer fixative (pH 7.4) at +4°C on rotator shaker. The cells were rinsed in 0.1 cocadilate buffer and incubated in same buffer over night at +4°C followed by washing in 5% sucrose in 0.05 M acetate buffer (pH 5.0). Amoeba cells were then fixed with 1% w/V OsO_4 in distilled water for 1 h at room temperature before blocks preparing for transmission electron microscopy.

RESULTS

At least one sp. of FLA were recovered from 59.1% of hospital water samples. While *Legionellae* were isolated only from 22% of the samples by standard culture method. The mean temperature of warm and cold water samples were 40.7°C (36-48°C) and 23.7°C (15-28°C), respectively. The mean pH of water samples was 5.95 (4.2-7.5). The free-chlorine concentration was obviously low in the samples 0-0.3 ppm (mean 0.2 ppm). One hundred and fifteen water samples were collected from hospitals scattered in major provincial cities (Fig. 1). After testing by PCR method, *Legionella* was detected from 17 further water samples which previously were recorded as *Legionella* negative. In another word totally, 39 (34%) of water samples were positive for *Legionella*. The colony size on primary culturing were too small after 48 h of incubation time and further 24 h were required for complete characterization (Fig. 2). The specificity of PCR protocol which used in the present study was obviously higher (34 compared to 21%) than standard culture method.

Different species of free-living amoeba were isolated from water samples depend on their nature and origin. The most prevalent FLA which recovered in characterized at species level were Acanthamoeba, Naegleria and Hartmanella (Fig. 3). FLA were isolated from totally 46% of whole water samples. The majority of FLA isolates were identified as either Acanthamoeba or Naegleria. Nearly 30% of samples were contaminated by both of *Legionella* and free-living amoeba (Fig. 4). The electron micrograph obviously showed the intra-amoebal multiplication of *Legionellae* (Fig. 5).

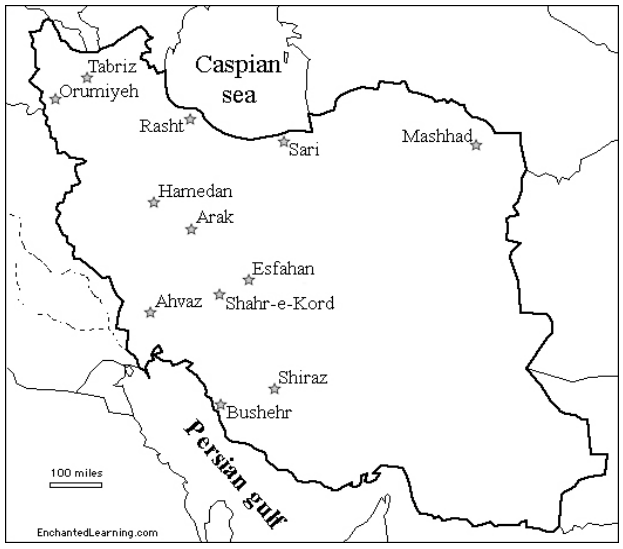

Fig. 1: National situated of hospital of the points of hospital water sampling

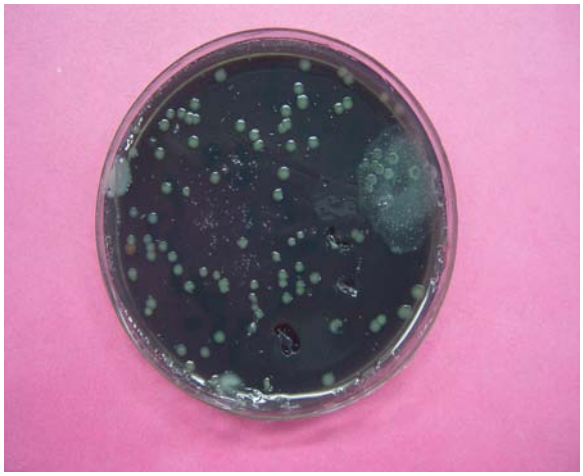

Fig. 2: *Legionella* colonies on BCYE agar after 72 h incubation time

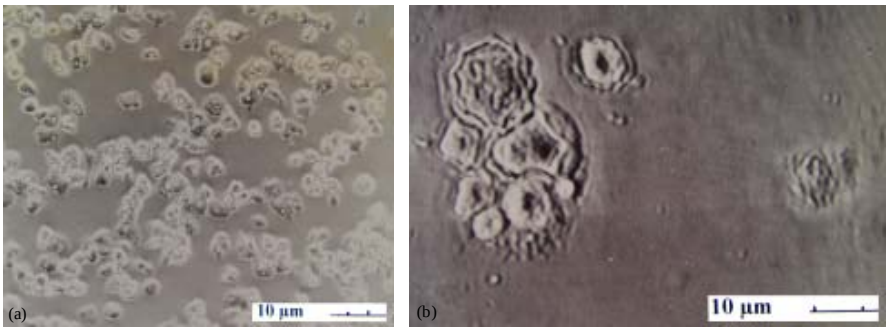

Fig. 3: Free-living amoebae (*Acanthamoeba* sp.) isolated from water sample. (a) Characteristic amoeba cysts and (b) Acanthamoeba trophozoites grow up within axenic culture

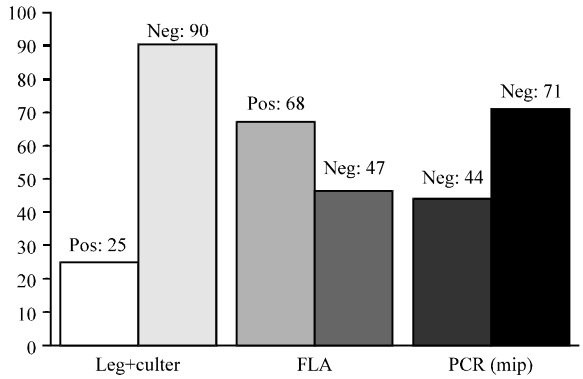

Fig. 4: The results of water sample examinations. First pair columns: Results of sample culturing for Free-Living Amoeba (FLA), Second pair columns: Sample culturing for *Legionellae* on standard media, Third pair columns: Polymerase Chain Reaction (PCR) results, Pos; Positive, Neg; Negative

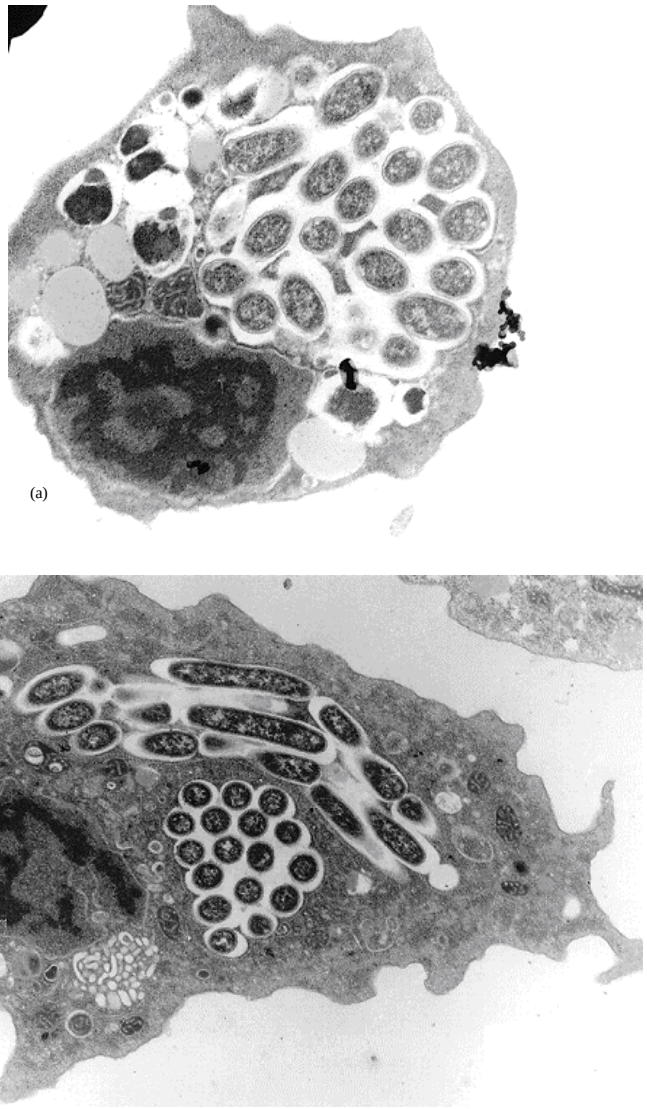

Fig. 5: Electron micrograph of hospital water isolated FLA (Acanthamoeba sp.) contaminated with *Legionella*. (a) Round structure (probably amoebae cyst) and (b) Trophozoite form. X4400

DISCUSSION

In this study, the co-existence of *Legionellae* and Free-living amoebae in hospital water networks were investigated. There was no recent history of any case of leginnaires' disease in some of the medical institutions. There are many similarities between the interactions of environmental protozoa with pathogenic bacterial species and those observed in mammalian macrophages. Since single-celled protozoa predate mammalian hosts, it is likely that interactions in environmental biofilms have selected for many of the bacterial virulence mechanisms responsible for human disease. Standard culture approaches and PCR methods were used to identify Amoebae Resistance *Legionellae* (ARL) in the present study. The contamination rate of hospital water samples with *Legionellae* by standard

culturing has been reported from 33-93% in different European countries (Miyamoto, Yamamoto *et al*., 1997; Kohler *et al*., 1999; Wellinghausen *et al*., 2001; Singh and Coogan, 2005), 63% in Turkey (Ozerol *et al*., 2005) and 36.6% in central of Iran (Attar *et al*., 2004). *Legionella* were cultured from approximately, 22% of water samples in present study. When we used PCR method to test water samples, *Legionella* genome were detected from 34% of samples which is within the range of contamination in regional and global scale. Pneumonia is an important cause of morbidity and mortality. Identification of the etiological agent is essential to select the appropriate antibiotic therapy. However, the etiology is identified in only about 50% of cases of community-acquired.

These so called Amoeba-Resisting Bacteria (ARB) resist the microbicidal effector mechanisms of amoebae (Greub and Raoult, 2004) and use the amoebae as a training ground for resistance to destruction by macrophages (Molmeret *et al*., 2005). Moreover, amoebae that are a reservoir for ARB (Amoeba Resistance Bacteria) are widely spread in the environment and in domestic water systems (Rodriguez-Zaragoza, 1994). The internalized bacteria may be protected from adverse conditions, particularly from agents used for water disinfection since amoebae are resistant to most of these disinfectants (Thomas *et al*., 2004), especially when they are encysted (Kilvington and Price, 1990). We recovered FLA from 60% of the hospital water samples totally. The prevalence of FLA was slightly, more than the prevalence found in previous studies (Rohr *et al*., 1998; Thomas *et al*., 2006), in which approximately 17 and 50% of the samples were found to be colonized by FLAs respectively. The upper rate of amoeba colonization that we observed could have been due to the low temperature of the hospitals water network compare with temperature of samples which they studied. In order to prevent colonization by *Legionella* sp., the temperature of hospital water supplies had been maintained at 65°C since 2000) (Blanc *et al*., 2005). In present study, the mean temperature of water samples was 43°C (range, 18 to 43°C).

Human infection occurs via inhalation of aerosols containing free bacteria (Bollin *et al*., 1985). It has also been suggested that infected amoebae could be the infectious particles that bring bacteria to the lungs (Rowbotham, 1986). Intracellular bacteria that grow poorly or not at all on the media used routinely in clinical diagnostic laboratories could be the agents responsible for pneumonia whose etiology is unknown. The candidates for these bacteria include intracellular colonizers of Free-Living Amoebae (FLA), such as *Legionella* (Fraser *et al*., 1977) and Parachlamydia (Greub and Raoult, 2002). Hospital water supplies often contain *Legionella* sp. and represent a potential source of nosocomial infection, especially for immuno-compromised patients or those in intensive care units. *Legionellae* were existed within approximately, 40% of environmental waters of Iran. The isolation of free-living amoebae within the majority of water samples indicated the relationship between *Legionella* and free-living amoebae. This data was accordance with previous reports (Hay *et al*., 1995; Singh and Coogan, 2005; Declerck *et al*., 2007). The exact correlations between water supplies contamination with *Legionellae* and existence of disease were reported previously (Kohler *et al*., 1999; Kowska *et al*., 2003; Nguyen *et al*., 2006). The most species of free-living amoeba identified in this study were Acanthamoeba, Naegleria, Hartmanella and Wahlkamphia which have well-known relationship with *Legionella* in water supplies (Bouyer *et al*., 2007). Therefore, even the *Legionella* negative samples could be considered as positive if amoeba isolated from them. Sampling of water from distribution systems of two hospitals. *Legionella* species have been a recognized and common pathogen of both nosocomial and community acquired pneumonia since 1976. The prevalence as reported in various studies ranges from 1-30%, but in a large number of studies, this etiology is one of the three most prevalent reported. Surveillance of splash water was performed in high-risk patient care areas. The fact that most of samples had pH slightly bellows the normal range, showed the excellent condition for *Legionella* biology within environmental water samples, even if their residual cholorine concentration were 0/2-0/4 mg mL^{-1}. Hospitals are usually points of congregation for persons at risk

for legionellosis especially legionnaires disease, beside providing suitable conditions for multiplication of *Legionellae*. The most samples that were screened in this study were collected from medical institutes, so the pathogen-free water should be provided for such high-risk patients. Among hospital facilities the dental unit is an environment that is at major risk of *Legionella* (Dutil *et al*., 2007) due to equipment such as the air/water syringe, the turbine, the micro-motors and the scalar which generate potentially harmful aerosols might be the source of exposure to *Legionella* sp. particularly in immuno-deficient patients and those affected by chronic diseases and also in dental personnel. Two positive samples of this study were belonged to dental clinics as well. Therefore, an examination of the extent of *Legionella* sp. contamination in the dental chairs waterlines and the incoming water supply of some public dental units is the subject of the present study.

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