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Table of Contents: Volume 11 Number 2 14 January, 2017

ARTICLES

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Review

Thermostable xylanase from thermophilic fungi: Biochemical properties and industrial applications

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Filamentous fungi have been investigated as producer of xylanases with relevant characteristics for application in different industrial sectors, such as bakery, beverage, biofuel, textile, animal feed, pharmaceutical, pulp and paper. Thus, this review will focus on biochemical properties and industrial use of thermostable xylanases produced by different filamentous fungi, as well as mechanisms of adaptation of thermophilic organisms to tolerate in high-temperature environments. These enzymatic properties of thermal and pH stability are crucial, especially in processes such as the manufacture of animal feed, pulp and paper industry. Reports on changes in enzyme structure, such as site-directed mutagenesis, insertion or substitution of amino acids, addition of disulfide bonds in the alpha helix or beta-sheet structure for improving the thermal stability will also be reported. However, strains of *Thermomyces lanuginosus* has been described as good producers of thermostable xylanases, as well as promising enzymes, because it does not require any change in structure to increase the tolerance to high temperatures.

Key words: Hemicellulase, thermostability, *Thermomyces*.

INTRODUCTION

The global enzyme market achieved a value of $4.8 billion in 2013 and it is expected to achieve a value of $7.1 billion in 2018; this translates into an expected annual growth of 8.2% between 2013 and 2018 (BBC Research, 2014). Therefore, the industrial market demand for thermostable enzymes and the search for microbial sources that are easily accessible and produce high levels of enzymes have constituted a challenge for researchers over the years (Araújo et al., 2008; Haki and Rakshit, 2003). In addition, there are also some reports of efforts to improve the thermal stability of enzymes by recombinant DNA technology (Haltrich et al., 1996; Beg et al., 2001).

Microorganisms such as fungi and bacteria are known to produce different types of enzymes that can act on complex components in the plant cell wall and hydrolyzing them into smaller molecules (Badhan et al., 2007). The plant cell wall consists of three major components: cellulose (40%), hemicellulose (33%) and lignin (23%), whose composition varies according to the

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plant source (Carvalho et al., 2009; Dhiman et al., 2008). The hemicellulose is a heteropolysaccharide component of the plant wall; it primarily comprises xylan, whose main chain is composed of residues of D-xylose joined by (1 → 4)-β-glycosidic linkages and the side chains by L-arabinofuranose, D-glucuronic acid or 4-O-methyl-D-glucuronic acid (Wakiyama et al., 2008; Adsul et al., 2011). The complete depolymerization of xylan requires the synergic action of several enzymes: endo-β-1,4-xylanase (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37) to hydrolyze the main chain; acetylxylan esterases (EC 3.1.1.72), α-L-arabinofuranosidases (EC 3.2.1.55), ρ-coumaric acid esterases (EC 3.1.1.-), α-glucuronidases (EC 3.2.1.139), and feruloyl esterase (EC 3.1.1.7-) to act on the side chains (Masui et al., 2012).

The xylanases belong to the family of glycosyl hydrolases (GH), which catalyze the hydrolysis of 1,4-linked β-D-xylosidic linkages in the main chain of xylan (Collins et al., 2005; Heinen et al., 2014). Based on the similarities between their amino acid sequences and the hydrophobic groups of their catalytic domains, fungal xylanases are classified into GH10 and GH11 families (Verma and Satyanarayana, 2012). GH10 family groups xylanases with higher molecular weight (approximately 40 kDa) pI acid and three dimensional $(\beta/\alpha)_8$ structures, while the GH11 family have lower molecular weights (approximately 20 kDa) basic pI, and three-dimensional β barrel structures (Beaugrand et al., 2004; Georis et al., 2000). Xylanases of both families (GH10 and GH11) have two glutamate residues conserved in their active sites and may possess carbohydrate binding modules (CBMs) or amino- or carboxyl-terminal regions (Sydenham et al., 2014).

The use of microbial xylan-degrading enzymes, which are stable in alkaline pH and at high temperatures, in the biobleaching of pulp has increased because they help to reduce the consumption of chlorinated compounds (Goluguri et al., 2012). However, these enzymes should possess characteristics such as high specific activity, resistance to chemicals (metal cations), and absence of cellulase activity (Walia et al., 2014). Thus, this review will focus on thermophilic fungi producing xylanase and its industrial applications, as well as mechanisms of adaptation of thermophilic organisms to tolerate the high-temperature environments. In addition, biochemical properties of xylanase produced by strains of *Thermomyces lanuginosus* will be emphasized.

In this context, the isolation of new thermophilic species capable of producing thermostable enzymes is advantageous (in relation to mesophilic microorganisms) since these enzymes do not require any structural modification to resist higher temperatures (Benassi et al., 2014). Many companies have commercialized enzymes derived from fungi, mainly for application in different industrial sectors. Table 1 shows some commercial enzymes, their characteristics and the associated industries.

THERMOPHILIC MICROORGANISMS AND ADAPTATION TO TOLERATE HIGH-TEMPERATURE ENVIRONMENTS

Organisms are generally grouped according to their optimum temperature range for growth: psychrophilic (below 15°C), mesophilic (15 to 45°C), thermophilic (between 45 and 80°C) and (above 80°C) hyperthermophilic (Taylor and Vaisman, 2010). Among the species of thermophilic fungi are found *T. lanuginosus* and *Talaromyces thermophilus*, which grow at 50°C (Romdhane et al., 2010; Singh et al., 2003), and *Rhizomucor miehei* and *Myceliophthora* species, which grow at 45°C (Badhan et al., 2008; Fawzi, 2011).

Adaptations to tolerate adverse conditions such as extreme pH, high salt concentrations, and high temperatures are inherent in a few microorganisms. Among them, high temperatures have greater influence on the maintenance of biological structures and biomolecules functions, and they are related to changes in the composition of the cytoplasmic membrane, DNA and proteins (Gomes et al., 2007; de Oliveira et al., 2015).

The cytoplasmic membranes of thermophilic organisms consist of saturated fatty acids that confer increased stability and physical and functional integrity, unlike those of mesophilic organisms, which have unsaturated fatty acids (Gomes et al., 2007). The maintenance of DNA structure is an essential factor for all organisms, especially for hyperthermophilic which survive in high temperature environments. According to Mehta and Satyanarayana (2013), some factors may combine to provide thermal stability to DNA in thermophiles, including high levels of K^+ and cyclic 2,3 diphosphoglycerate (2,3-DPG) that has been detected in the cytoplasm of thermophilic methanogens such as *Methanothermus fervidus*, *Methanothermus sociabilis* and *Methanopyrus kandleri* (Gomes et al., 2007; Scholz et al., 1992). Marguet and Forterre (1998) reported that 2,3-DPG prevents depurination or depyrimidation of DNA, which causes mutations at high temperatures. In addition, all hyperthermophilic organisms produce a different form of reverse DNA topoisomerase called DNA gyrase, which introduces positively supercoiled DNA. The positive supercoiling promotes greater DNA resistance to thermal denaturation (Gomes et al., 2007; López-García, 1999). The molecular chaperones are also important for thermophilic adaptation of microorganism. They result in the folding and refolding of proteins, preventing possibly irreversible protein denaturation (Conway and Macario, 2000). Nevertheless, the explanation of the mechanism involved in the survival of microorganisms, especially eukaryotes, at high temperatures is still not fully understood. On the other hand, there are many reports on biochemical characteristics of thermostable xylanases produced by thermophilic organisms (Table 2).

Xylanases derived from thermophilic fungi have

Table 1. Commercial enzymes and their applications.

Commercial product	Enzyme	Microbial source	Industry
ACCELLERASE® 1500	Exoglucanase, endoglucanase, hemicellulase and β-glucosidase	*Trichoderma reesei* genetically modified	Cellulosic ethanol
AlternaFuel® CMAX™	Cellulase	*Myceliophthora thermophila*	Biofuels
Biolase Alfa	Alpha-amylase	*Aspergillus oryzae*	Baking industry
Biolase MAXI	Xylanase	*Aspergillus niger*	Baking industry
Cellic® CTec3	Cellulase/Hemicellulase	NR	Cellulosic ethanol
Dyadic® Xylanase 2XP CONC	Endo-1.4-β-D-xylanase	*Trichoderma longibrachiatum* (*Trichoderma reesei*)	Food, brewery and application in animal feed
EXCELLENZ™ P 1250	Protease	NR	Laundry
FibreZyme® G4	Cellulase	*Myceliophthora thermophila*	Pulp and Paper
Fungamyl®	Alpha-amylase	NR	Bakery
LAMINEX®	β-glucanases/Xylanases	NR	Brewery
Lipex®	Lipase	NR	Laundry

exhibited molecular weights ranging from 21 to 47 kDa and a broad isoelectric point range of 3.5 to 8.7 (Table 2). There is a predominance of enzymatic activity in alkaline pH and high temperatures (up to 60°C). Most of the xylanases exhibit stability in a wide pH range (5.0 to 9.0), as *Thermoascus aurantiacus* KKU-PN-I2-1xylanase, which maintained activity in alkaline pH (9.0) for 24 h (Table 2). In addition, *R. miehei* NRRL 3169 xylanase showed high thermostability, retaining 100% of activity after 60 min of incubation at 70 to 75°C (Table 2).

THERMOSTABLE XYLANASE AND ITS PROPERTIES

Thermostability studies of some fungal enzymes have been described in the literature, but this attribute is still not fully understood. Higher thermal stability is one of the fundamental requirements for the application of an enzyme in industrial processes; it increases the efficiency of enzyme. Therefore, searching for thermostable enzymes or improve the thermostability of enzymes has been the priority for researchers over the years (Nirmal and Laxman, 2014). The factors affecting thermostability are important for understanding the functions of proteins and their use in various industries (Ruller et al., 2008). The advantages of employing enzymes with high optimum temperatures in biotechnological processes or biocatalytic conversions industrial include the lower risk of microbial contamination by common mesophiles, the improvement of substrate solubility, increased reaction rates and decreased viscosity (Joo et al., 2011; Haki and Rakshit, 2003). The tolerance of the enzymes to high temperatures for long periods may be associated with their conformational structures, composition and/or amino acid sequences, and the origins of the enzymes (Gomes et al., 2007; Techapun et al., 2003). In general, a strategy for obtaining thermostable enzymes is search in organisms that grow in high temperature environments because their enzymes are more thermostable than those from mesophilic (Techapun et al., 2003). Several authors have reported thermostable xylanases obtained from thermophilic fungi, such as, *Humicola brevis* var. *thermoidea* (Masui et al., 2012), *Paecilomyces themophila* J18 (Yang et al., 2006) and *T. aurantiacus* RCKK (Jain et al., 2015). Most of these fungal xylanases thermostable belong to GH10 and GH11 family, and enzymes belonging to the same family, they present the same structure and amino acid sequence, and thus the thermal stability also did not differ (Collins et al., 2005; You et al., 2010).

It is also possible to obtain thermostable enzymes by the improvement of characteristics through small alteration in enzyme structure. These include site-directed mutagenesis (Wang et al., 2014; Zheng et al., 2014; Xie et al., 2011), replacing the N-terminal region of a mesophilic xylanase by N-terminal region of thermophilic organisms (Zhang et al., 2010) and the addition of disulfide bridges in the N-terminal region of the α-helix. Hakulien et al. (2003) analyzed crystal

Table 2. Biochemical properties of xylanases produced by thermophilic fungi.

Strain	Molecular mass (kDa)	pI	Optimum pH	Optimum temperature (°C)	pH stability	Termal stability	References
Malbranchea cinnamomea strain S168	43.5	4.37	6.5	80	4.0-10.5: 30 min	30-65°C: 100% - 30 min	Fan et al., 2014
Malbranchea flava							
MFX I	25.2	4.5	9.0	70	9.0 and 60°C: 50% - 240 min		Sharma et al., 2010
MFX II	30	3.7	9.0	70	9.0 and 60°C : 46% - 240 min		
Myceliophthora sp. IMI 387099							
Xyl IIa	47	~3.5	8.0	70	9.0 and 50°C: ~80% - 60 min		
Xyl IIb	41	~4.8	9.0	60	9.0 and 50°C: 80% - 180 min		Badhan et al., 2008
Xyl IIc	30.1	5.2	7.0	80	7.0 and 50°C: ~100% - 30 min		
Remersonia thermophila CBS 540.69	42	NR	6.0	65	5.5: >85% - 7.5 min	50°C: 50% - 30 min	McPhillips et al., 2014
Rhizomucor miehei NRRL 3169	27	NR	5.5-6.0	75	5.0 and 6.5: 90% - 60 min	70-75°C: 100% - 60 min 40°C: >95% - 240 min	Fawzi, 2011
Scytalidium thermophilum ATCC No. 16454	21	8.6	6.5	65	6.0-8.0: 360 min	50°C: 85% - 120 min 60°C: 50% - 120 min	Kocabaş et al., 2015
Thermoascus aurantiacus KKU-PN-I2-1	27	7.2	9.0	60	7.0-9.0: >70% - 24h	50°C: >70% - 90 min	Chanwicha et al., 2015
Recombinant xylanase							
MpXyn10A of *Malbranchea pulchella* express in *Aspergillus nidulans*	40.747	NR	5.5	80	NR	65°C: 85% - 1440 min 70°C: 80% - 60 min	Ribeiro et al., 2014
Xyn11B of *Humicola insolens* Y1 express in *Pichia pastoris*	29.1	8.7	6.0	50	5.0-9.0: ~80% - 60 min	40°C: >40% - 30 min	Shi et al., 2015
XynA of *Paecilomyces thermophile* express in *Pichia pastoris*	29	NR	7.0	75	4.0-11.0: >80% - 30 min	30-70°C: >80% - 30 min	Fan et al., 2012

NR: Not reported.

structures of 12xylanases belonging to family 11 (GH11), and concluded that the xylanase structures of mesophilic and thermophilic organisms were similar and that minor modifications altered thermostability. Such modifications included larger numbers of amino acid residues in beta sheets and the stabilization of the alpha-helix region; higher proportions of threonine:serine; increasing charged amino acid residues such as arginine, leading to an improvement in polar interactions; more compact structures and more aromatic residues or pairs of ions in protein surfaces. Also, according to You et al. (2010), the replacement of the cysteine at site 201 of the xylanase (GH11) improved its thermostability due to the strong hydrophobic interaction with the cysteine at site 50. Therefore, disulfide bond formation and hydrophobic interactions contributed to the rise in the thermal stability of xylanase. Similarly, Song and colleagues (2015) reported the importance of the amino acid of the N-terminal region of the *Aspergillus niger* (Xyn10A_ASPNG) xylanase for improving its thermostability, and the thermal

inactivation half-life ($t_{1/2}$) at 60°C was prolonged by 30 times in comparison with wild-type enzyme.

APPLICATION OF THERMOSTABLE XYLANASES IN INDUSTRIAL PROCESSES

The application of enzymes in industry has grown over the years. Some enzymes, such as xylanases, lipases, cellulases, proteases, amylases and phytases, have been used in feed industry to reduce the viscosity of the food and improve the absorption of nutrients in the digestive tracts of animals (Polizeli et al., 2005). These enzymes may act when the feeds are being processed (for example, when thermostable xylanases are added before the pelletization process (70-95°C), transported, and stored; they can also facilitate digestion in the gastrointestinal tract of the animal (Pariza and Cook, 2010; Collins et al., 2005).

Furthermore, xylanases are used in the food industry. For example, in the production of bread, enzymes are added to improve its softness and shelf life (Sharma and Kumar, 2013). A study by Jiang et al. (2005) evaluated the effect of xylanase purified from *T. lanuginosus* CAU44 on the quality of bread and its staling rate during storage and reported that thermostable xylanase could be used in the bakery industry. Similarly, the potential of xylanase XYNZG from *Plectosphaerella cucumerina* for baking by heterologously expressed in *Kluyveromyces lactis* was studied. They obtained improvement in sensorial characteristics, volume, texture, and handling time (6.5 to 6.0 min) (Zhan et al., 2014). The purified xylanase from *Remersonia thermophila* CBS 540.59 (Rtl) also showed increased loaf volume by 8%, softness (19.6%) and decreased in 20.4% hardening of the bread after four days of storage compared to the control (McPhillips et al., 2014). The enzyme cocktail that the thermophilic fungus *T. aurantiacus* (CBMAI 756) produced was used (35 U of xylanase/100 g of flour), resulting in the increase of loaf volume (by 22%), the reduction in crumb firmness (by 25%), and amylopectin retrogradation (by 17%) (Oliveira et al., 2014).

Xylanases are also used in the beverage industry together with pectinases, cellulases and amylases, which act to recover flavor, reduce viscosity and turbidity, and stabilize fruit pulp (Polizeli et al., 2005). Thermoacidophilic xylanase from *Penicillium pinophilum* C1 was employed in the brewing industry. The enzyme has improved the filtration rate to 22.3% and the viscosity of wort to 5.0% with 40 U of xylanase (purified XYN10C1) (Cai et al., 2011).

Moreover, the addition of a higher dose of the enzyme (80 U) improved these parameters, resulting in the filtration rate to 26.7% and the viscosity of wort to 9.8%. Similarly, the recombinant xylanase from *Gloeophyllum trabeum* reduced the specific filtration rate and viscosity of wort to 17.2 and 7.1%, respectively using 40 U of GtXyb10 enzyme, but it was more effective with 80 U of the enzyme, reducing the filtration rate to 31.3% and the viscosity of wort to 12.8% (Wang et al., 2016).

In the pulp and paper industries, thermophilic, alkalophilic, and cellulase-free xylanases have been used in the biobleaching of pulp. They facilitate the depolymerization of xylan, leading to the formation of pores and giving chlorinated reagents the necessary access for the removal of lignin from the wood pulp. Thus, these enzymes provide eco-friendly alternative for the effective bleaching of pulp, reducing the use of toxic chlorine compounds (Christopher et al., 2005; Subramaniyan and Prema, 2000; Kanwar and Devi, 2012; Sharma et al., 2015). Thus, several studies have focused on the use of enzymes in the pretreatment of pulp. For instance, the cellulase-free xylanase from *Trichoderma viride* was used on kraft pulp from *Eucalyptus grandis*, resulting in a decrease in its Kappa number and its maintenance of viscosity compared to those of the control, with parameters (enzyme dose, time, temperature, and pH). The results indicated that the enzyme showed potential for use in the pulp and paper industry (Fortkamp and Knob, 2014). Similar studies by Guimarães et al. (2013) using a xylanase from *Aspergillus aculeatus* var *aculeatus* in the pretreatment of *E. grandis* pulp, and by Silva et al. (2016) described the effective use of xylanase from *Penicillium crustosum* to bleach the kraft pulp of *E. grandis*, obtained a significant reduction in Kappa number (5.27 points) corresponding to a 35.04% Kappa efficiency. Although, there are some reports in the literature on the use of thermostable microbial xylanases, especially in the paper industry, there is still a deficit of enzymes with stability at high temperatures and alkaline pH. Thus, the search for new strains of fungi capable of producing thermostable enzymes at high temperatures and alkaline pH remains relevant.

Furthermore, alkaline xylanases are used in the detergent industry. They are additives in the formulation of detergents and, thus, improve the removal of stains of vegetable origin (Kamal et al., 2004). In the textile, enzymes act on cotton cleaning (cotton biopolishing), in order to improve the physical characteristics of the tissue, to assist in the removal of non-cellulosic materials and facilitate alkaline extraction step to improve the access of compounds chemicals to the fiber, leading to a reduction in consumption of these agents and possible environmental damage (Battan et al., 2012; Csiszár et al., 2006).

T. lanuginosus

T. lanuginosus is a thermophilic filamentous fungus, synonymous with *Humicola lanuginosa*. The *Thermomyces* genus includes four species: *T. lanuginosus* Tsiklinsky, which was first isolated

Table 3. Biochemical properties of xylanase produced by *T. lanuginosus*.

Strains	Molecular mass (kDa)	pI	Optimum pH	Optimum temperature (°C)	pH stability	Thermal stability (°C)	References
SSBP	23.6	3.8	6.5-7.0	70-75	5.0-12.0	60-75	Lin et al. (1999)
DSM 10635	25.5	3.7	6.5	70	4.0-9.0	50-100	Xiong et al. (2004)
THKU-49	24.9	3.7	6.0	70	NR	50-70	Kchucharoenphaisan et al. (2008)
THKU-9	24.9	3.7	6.0	70	NR		
SS-8	23.79	3.9	6.0	60	5.0-11.0	60	Shrivastava et al. (2013)
CBS 288.54	26.2	NR	7.0-7.5	70-75	6.5-10	40-85	Li et al. (2005)
ATCC 46882	26.3	3.7	6.0-6.5	75	4.0-10	45-60	Bennett et al. (1998)
DSM 5826	25.5	4.1	6.5	60-70	4.0-12.0	65-70	Cesar and Mrša (1996)
CAU 44	25.6	NR	6.2	75	5.6-10.3	30-80	Jiang et al. (2005)
195	22	NR	NR	NR	3.0-10.0	60-100	Gaffney et al. (2009)

NR: Not reported.

from garden soil in 1899 (Pugh et al., 1964); *Thermomyces ibadanensis* Apinis & Eggins, a thermophilic and lipolytic fungus isolated from palm fruit in 1966 (Apinis and Eggins, 1966); *Thermomyces stellatus* (Bunce) Apinis, a thermophilic species found in hay 1961 (Bunce, 1961); and *Thermomyces verrucosus* Pugh, Blakeman and Morgan-Jones, a mesophilic species discovered in 1964 (Pugh et al., 1964). *T. lanuginosus* grows at temperatures above 45°C and is initially white in color, becoming dark brown after maturation (Khucharoenphaisan and Sinma, 2010).

There are many reports of strains of *T. lanuginosus* with high ability to produce thermostable enzymes using inexpensive carbon sources such as sorghum straw (Singh et al., 2000; Sonia et al., 2005) and corn cobs (Winger et al., 2014). These alternative carbon sources can reduce the investment in the production of enzymes. Moreover, the reuse of agricultural waste can contribute to ecologically correct efforts to reduce the disposal of materials in the environment and convert raw materials into valuable products such as biofuels (Howard et al., 2003).

T. lanuginosus is an interesting fungus because it can produce enzymes of industrial interest such as invertase (Chaudhuri and Maheshwari, 1996), β-xylosidase (Corrêa et al., 2016), chitinase (Khan et al., 2015; Chen et al., 2012; Zhang et al., 2015), protease (Li et al., 1997), inulinase (Flores-Gallegos et al., 2015), esterase (Li et al., 2014), amylase (Kunamneni et al., 2005), glucoamylase (Gonçalves et al., 2008), α-galactosidase (Rezessy-Szabó et al., 2007), and the two most reported enzymes, lipase (Wang et al., 2015; Ávila-Cisneros et al., 2014; Fang et al., 2014) and xylanase (Jiang et al., 2015; Stephens et al., 2014; Shrivastava et al., 2013; Manimaran et al., 2009). Furthermore, *T. lanuginosus* is known for producing cellulase-free xylanases (Manimaran et al., 2009; Li et al., 2005; Damaso et al., 2002). This characteristic free cellulase is essential for the application of xylanase in pulp and paper industry because it prevents degradation of the cellulose (Beg et al., 2001). Furthermore, xylanases from *T. lanuginosus* has been reported to belong to family 11 glycoside hydrolases (Wang et al., 2012; Gruber et al., 1998).

According to Gruber et al. (1998), the crystal structure of the *T. lanuginosus* xylanase is a compact globular protein consisting of two highly twisted β-sheets and one α-helix, and it is surrounded by water molecules on its surface as well as one (O201) on the inside. Moreover, the enzyme has a disulfide bond that is absent in most other xylanases of the GH11 family. This could be related to electrostatic interaction through the ion pairs and could explain their tolerance to higher temperatures. Table 3 summarizes some biochemical characteristics of xylanases from different strains of *T. lanuginosus*. The xylanases from different strains of *T. lanuginosus* exhibit low molecular weights (22 to 26 kDa), facilitating their passage through hemicellulose networks and resulting in the improvement of enzymatic hydrolysis (Juturu and Wu, 2012), enzymatic activity at high

temperatures (60 to 75°C), and thermal stability (30 to 100°C). This ensures that they can be used at high temperatures and remain stable in a wide pH range (3 to 12), making them relevant in different biotechnological processes (Chen et al., 2014; Mamo et al., 2009).

CONCLUSION

Despite the biotechnological advances of recent decades, most studies have focused on the production, purification, biochemical characterization, and regulation of thermostable fungal xylanases. Although there are some reports in the literature regarding the use of thermostable microbial xylanases, especially in the paper industry, they lack stability at high temperatures and in alkaline pH. Some studies have shown that small changes in enzyme structure, through site-directed mutagenesis, insertion or substitution of amino acids, or addition of disulfide bridges to stabilize alpha-helix structures or beta sheets, result in the improved thermal stability of the enzyme. On the other hand, there are many strains of *T. lanuginosus* that are good producer of thermostable xylanases with activity at high temperatures (60 to 75°C) and in a wide range of thermal stability (30 to 100°C). Xylanases with this property would be particularly relevant and advantageous to detergents industry and pulp and paper, because it does not require any change in structure to increase the tolerance to high temperatures.

Conflict of Interests

The authors have not declared any conflict of interests.

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Escherichia coli bacteremia: Clinical features, risk factors and clinical implication of antimicrobial resistance

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Escherichia coli **is an important cause of both community acquired (CA) and hospital acquired (HA) bacteremia. A prospective study was conducted at a tertiary care University Hospital from January, 2012 to July 2014, to compare the clinical features, risk factors, outcomes and antimicrobial resistance between *E. coli* bacteremia acquired from the community (CA) versus *E. coli* bacteremia acquired from the hospital (HA). Clinical and laboratory data of 171 adult patients with at least one positive blood culture of *E. coli* were analyzed. Data were collected from patients with significant blood stream infection, using medical and laboratory record files and information from treating medical staff. The overall incidence of extended spectrum beta lactamase (ESBL) infection was high, 67/171 (77.4%). Thirty-eight (40.9%) of the CA isolates were found to produce ESBL, while 28 (35.9%) of the HA isolates were ESBL producers. Patients with CA bacteremia tend to be older than those with HA bacteremia (0.003). Neoplastic diseases (hematological malignancy (<0.001), solid tumors (<0.001)), renal transplantation end stage renal disease (ESRD) (<0.006), and wound infection (<0.001) were the most commonly associated conditions in patients with HA bacteremia. Patients from the community are more likely to present with UTI (<0.001), fever and pyelonephritis (0.001). Both CA and HA *E. coli* isolates showed the highest sensitivity to imipenem, meropenem and amikacin followed by gentamicin and tazocin. The CA isolates are more susceptible to amikacin, tazocin and ciprofloxacin than the HA isolates. No significant difference in the mortality rate between patients with CA bacteremia and patients who acquire the bacteremia in a hospital setting (0.836) was observed. Clinicians need to be aware of the risk factors and changing pattern of antimicrobial resistance of this pathogen and should consider adequate empirical therapy with coverage of these pathogens for patients with risk factors**

Key words: *Escherichia coli,* community acquired, hospital acquired, bacteremia, blood stream infection.

INTRODUCTION

Escherichia coli are part of the normal gastrointestinal flora and a leading cause of Gram negative bacteremia (Tenaillon et al., 2010). Sepsis and septic shock caused by *E. coli* and other Gram-negative bacteria is due to the inflammatory response activated by endotoxin (lipopolysaccharide) present in the Gram-negative cell

wall (Johnson et al., 2006). Blood stream infection (BSI) in developing countries is a serious issue that is rarely addressed in the scientific literature (Aiken et al., 2011). Bloodstream infection (BSI) due to extended-spectrum β-lactamase (ESBL) *Enterobacteriaceae* has emerged as a major cause of in-hospital mortality (Hyle et al., 2005; Pitout and Lauplan, 2008). The spread of community-acquired and hospital-acquired (nosocomial) bacteremia cause by *E. coli* imposes a major health burden. However, only few regional information is available on the differences between hospital-acquired and community acquired *E. coli* bacteremia (Hoenigl et al., 2014). Community and hospital spread of *E. coli* producing extended-spectrum beta-lactamases has increasingly been reported, most notably *E. coli* producing CTX-M strains (Woodford et al., 2004). This poses significant challenges to clinicians caring for patients presenting to hospital with suspected sepsis as empiric antibiotic treatment is often targeting presumed, antibiotic-susceptible community organisms (Rodriguez-Bano et al., 2006; Tumbarello et al., 2008). Accordingly, this study was conducted to assess any demographic variation in the incidence, the clinical characteristics, risk factors and antimicrobial-resistance trends of community-associated (CA) and hospital associated (HA) *E. coli*-bacteremia, presenting to the hospital. To the best of the authors' knowledge, there are no other studies comparing the epidemiology and risk factors between the community-acquired and health-care associated *E. coli* bacteremia from the Gulf region.

METHODS

Patients

This study was conducted at King Khalid University Hospital, a 2500 bed major teaching hospital in Riyadh that provides both primary and tertiary medical care. From January 1, 2012 to July 30, 2014, adult patients (>14 years old) with at least one positive blood culture of ESBL-producing *E. coli* and non-ESBL-producing *E. coli* were reviewed. Only the first episode of bacteremia in each patient was included in the analysis.

Definitions and data collection

Data were prospectively collected from patients with significant blood stream infections using daily review of blood culture results, patients' medical record files, information from treating medical staff and by a computerized method using the blood culture register numbers in the microbiology laboratory of each positive case. Standardized data forms were used to record demographic details including underlying diseases, hospital unit, and exposure to the healthcare system in the previous year, site of infection, ESBL production in organisms isolated from culture samples, clinical progress and mortality. Patients were divided into two groups based on the onset of bacteremia. Bacteremia with *E. coli* detected within the first 48 h of hospitalization was classified as "community-onset" according to the US Centers for Disease Control and Prevention definition and hospital acquired *E coli* infection was defined as an infection that occurred > 48 h after admission to the hospital, or an infection that occurred < 48 h after admission of patients that had been transferred from another hospital or nursing home (National Committee for Clinical Laboratory Standards, 1999) and were further classified into community-acquired or health care associated infections (modified from the study of Siegman-Igra et al., 2002). The former definition represents truly community-acquired infection, while the latter consists of infections in patients recently discharged (≤ 6 months), infections associated with invasive procedures performed earlier, or at the time of admission and infections in patients admitted from nursing homes. *E. coli* bacteremia was defined as the isolation of *E. coli* from ≥ 1 set of aseptically inoculated blood culture bottles. In patients with clinical features compatible with systemic inflammatory response syndrome. Patients were classified as immunosuppressed if neutropenia (defined as < 1,000 polymorphonuclear neutrophils cells/mm^3), hematologic malignancy, corticosteroid therapy (equivalent to > 20 mg prednisolone/day) for at least 2 weeks, and/or cancer chemotherapy or radiation therapy were documented within 30 days of the onset of bacteremia. Patients with serum creatinine level > 3 mg/dL, or under dialysis, before the onset of bacteremia were considered to have chronic renal insufficiency.

Identification and antimicrobial susceptibility testing

Isolates of *E. coli* were identified by standard microbiologic methods in the microbiology laboratory using an automated identification system (Vitek System; bioMe´rieux). Susceptibilities to antimicrobial agents (ampicillin, amoxicillin/clavulnate, cefradine, cefuroxime, ceftriaxone, cefotaxime, ceftazidime, cefipem, ciprofloxacin, imipenem, meropenem, gentamicin, amikacin piperacillin/ tazobactam, trimethoprim/sulfmethoxazole) were determined by use of an automated susceptibility testing system (Vitek 2 System; bioMe´rieux). ESBL production was detected and interpreted using CLSI criteria for broth dilution in accordance with the Clinical and Laboratory Standards Institute standards (Wayne, 2005).

Statistical analysis

All statistical analyses were performed using the SAS software package (version 9.1; SAS Institute Inc., Cary, NC, USA). For univariate analysis, categorical variables were compared using χ^2 or Fisher's exact test and continuous variables were analyzed with Student's *t* test or Mann- Whitney *U* test. A p value < 0.05 was considered to be statistically significant, and all probabilities were two-tailed.

RESULTS

During the study period, 171 adult patients with *E. coli* bacteremia were analyzed. Of these, 93 (54.4%) were community-acquired *E. coli* bacteremia and 78 (45.6%) were hospital-acquired *E. coli* bacteremia (Table 1).

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Table 1. Classification of 171 patients with *E. coli* bacteremia.

	Community acquired *E. coli*	Hospital acquired *E. coli*
ESBL	38 (40.9%)	28 (35.9%)
Non-ESBL	55 (59.1%)	50 (64.1%)
Total	93	78

Table 2. Clinical characteristics of 171 patients with *E. coli* bacteremia.

Characteristics	Community acquired (n=93)	Hospital acquired associated (n=78)	p Values
Age (mean + SD), years	58.8 ± 21.8	48.2 ± 24.6	0.003
Sex (M/F)	34 / 59	41 / 37	0.047
Underlying disease			
Diabetes mellitus	5 (5.3%)	3 (3.8%)	0.637
liver cirrhosis/ biliary tract disease	1 (1.1%)	4 (5.1%)	0.114
ESRD/ post-transplant	2 (2.1%)	10 (12.8%)	0.006
Solid tumor	0	15 (19.2%)	<0.001
Hematological malignancy	1 (1.1%)	11 (14.1%)	<0.001
Heart disease	2 (2.1%)	4 (5.1%)	0.286
RTA	0	1 (1.3%)	0.271
Clinical presentation			
Urinary tract infection	20 (21.3%)	2 (2.6%)	<0.001
Fever/Pyelonephritis	41 (43.6%)	16 (20.5%)	0.001
Septic shock/hypotension	9 (9.6%)	4 (5.1%)	0.272
Wound infection/diabetic foot	1 (1.1%)	11 (14.1%)	0.001
Vomiting/diarrhea	5 (5.3%)	0	0.039
ESBL	39 (41.5%)	28 (35.9%)	0.454
Mortality	24 (25.5%)	21 (26.9%)	0.836

Demographic and clinical characteristics of patients are shown in Table 2. Patients with community acquired *E coli* bacteremia tend to be older than those with hospital-acquired infection (0.003); they were more than 55 years old and were mostly female. Hematological malignancy (<0.001), solid tumors (<0.001), renal transplantation, end stage renal disease (ESRD) (<0.006), and wound infection including diabetic foot infection (<0.001) were associated with hospitalization and development of *E. coli* bacteremia. Among patients with malignancy, hematological malignancy was found to be a significant risk factor for acquisition of E coli bacteremia in hospitalized patients (14.1%) (<0.001). Patients from the community are more likely to present with urinary tract infection (<0.001), fever and pyelonephritis (0.001) or vomiting and diarrhea (0.039). Among the 78 hospitalized patients, oncology (30.8%), medicine (28.2%), and critical care (23.1%), were the commonest specialists at the onset of bacteremia (Table 3). The overall incidence of ESBL infection was high, 67/171 (77.4%). Thirty-eight (40.9%) of the community acquired isolates were found to produce ESBL, while 28 (35.9%) of the hospital acquired isolates were ESBL producers. There was no significant difference in acquiring infection with ESBL *E. coli* between patients from the community and hospitalized patients. Both community-acquired and hospital-acquired *E. coli* isolates showed the highest sensitivity to imipenem, meropenem and amikacin followed by gentamicin and piperacillin/tazobactam (Figure 1). The sensitivity pattern of ESBL producing *E. coli* of the community-acquired and hospital-acquired isolates is shown in Figure 2. Meropenem and imipenem are the most sensitive antimicrobial agents followed by the amikacin and piperacillin/tazobactam. The community-acquired isolates are more susceptible to amikacin, piperacillin/tazobactam and ciprofloxacin than the hospital-acquired isolates. No significant difference was observed in the mortality rate between patient who acquire the bacteremia from the community or those who acquire the bacteremia in a hospital setting (0.836)

Table 3. Admission characteristic of 78 hospitalized patient with *E. coli* bacteremia.

Characteristics	N (%)
Age , median years (range)	54.5
Male sex	41 (52.6%)
Hospital ward	
Medical service	22 (28.2%)
Surgical service	14 (17.9%)
Intensive care unit	18 (23.1%)
Oncology	24 (30.8%)

Table 4. Mortality among ESBL and non-ESBL patients.

	ESBL	non-ESBL	Total
Community acquired	11 (45.8%)	13 (61.9%)	24
Hospital acquired	13 (54.2%)	8 (38.1%)	21
Total	24	21	45

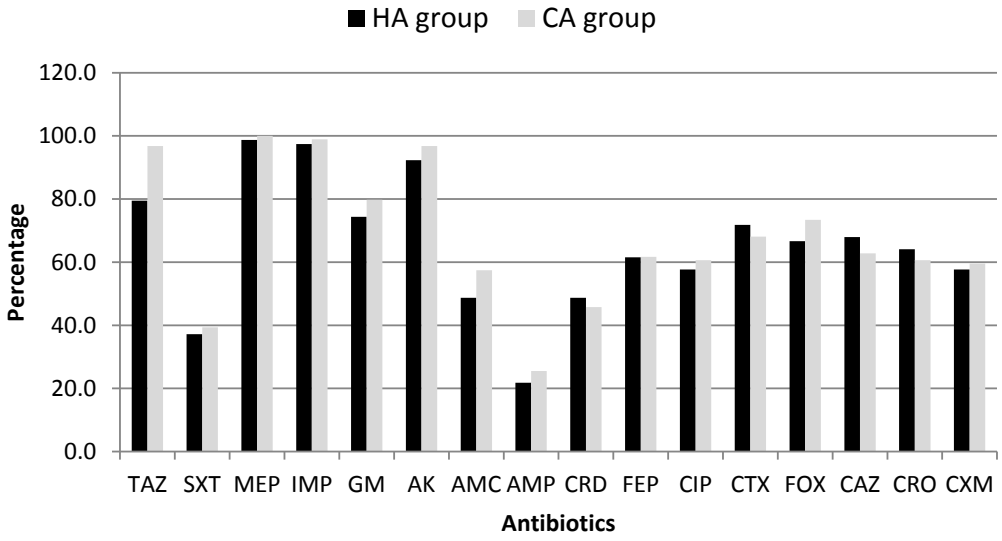

Figure 1. Percentage sensitivity of community acquired (CA) and hospital acquired (HA) *E. coli* to antimicrobial agents.

(Table 4).

DISCUSSION

E. coli-blood stream infection is a major cause of morbidity and mortality with a relatively high associated population burden (Pitout et al., 2004; Uslan et al., 2007; Williamson et al., 2013). Little data exists on the demographic variation and potential risk factors between CA and HA *E. coli* blood stream infection (Pitout et al., 2004; Rodríguez-Baño et al., 2010) such population-based demographic information is important in implementing strategies for treatment and prevention of these serious infections. There were many studies from the Saudi Arabia region that determine the prevalence of bacterial pathogens isolated from all specimen types including blood and assessed the multi-drug resistant rates of ESBLs among *Enterobacteriaceae*. The prevalence between 4.8 and 15.8% have been reported

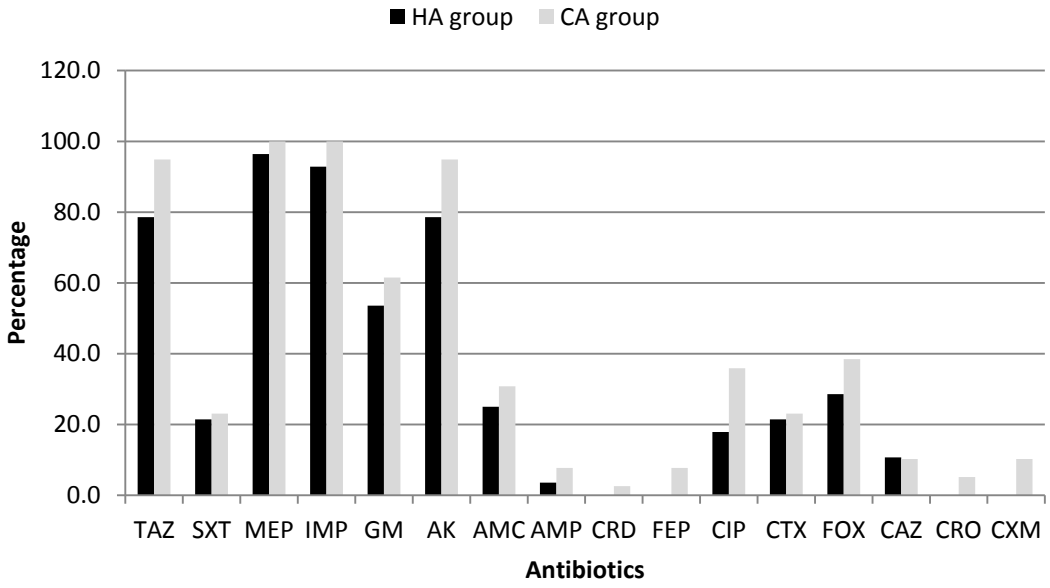

Figure 2. Percentage sensitivity of community acquired (CA) and hospital acquired (HA) ESBL producing *E. coli*.

from Saudi Arabia with the finding of the lowest frequency rates of ESBL producers in the eastern region and the highest frequency was observed in the central region (El-Khizzi and Bakheshwain, 2006; Kader and Kumar, 2004; Masoud et al., 2011; Rodríguez-Bano et al., 2009). In two studies (El-Khizzi and Bakheshwain, 2006; Khanfar et al, 2009) from the Arabian Gulf region, ESBL detection in *Enterobacteriaceae* was described. In the first study (El-Khizzi and Bakheshwain, 2006), different patient populations with nosocomial and community-acquired infections were assessed, the majority (83%) of the ESBL-producing isolates were *E. coli*. ESBL producers were significantly higher among isolates from in-patients, 15.4% as compared to those from out-patients, 4.5%. Urine was the most common specimen for the isolation of ESBL pathogens among in-patients and out-patients. In the second study from Qatar, Khan et al., 2010 reported the occurrence of resistant Gram-negative organisms in 63.1% of bacteremia patients with the following prevalence: ESBL-producing *p0ki9* (34%), followed by *Klebsiella* spp. (13.7%) and finally *Pseudomonas aeruginosa* (7.4%). A recent study on the characteristics of hospital-acquired and community-onset blood stream infections from Austria (Hoenigl et al., 2014), *E. coli* followed by *Staphylococcus aureus* were the most frequently isolated pathogens. This study has shown that, ESBL producing *E. coli* is an important cause of bloodstream infection presenting from both, the community and the hospital settings (40.9 and 35.9%, respectively). The overall incidence of *E. coli* ESBL bacteremia in this study is high, higher than the rate reported by Memom et al., 2009 and Kang et al., 2013**,** from the eastern region of Saudi Arabia (31%), and from Korea (33%), respectively. In another retrospective study from Taiwan [6], of 404 episodes of community-onset *E. coli* bacteremia, the frequency of ESBL producers was 4.7%. This rate is considerably lower than the rate found in our study. The differences in risk factors between CA and HA bacteremia was also identified. Patients with community acquired *E coli* bacteremia tend to be older than those with hospital-acquired infection and are mostly females. This finding is in agreement with a population-based incidence and comparative study (Williamson et al., 20113) of community-associated and healthcare-associated *E. coli* bloodstream infection from New Zealand, which revealed that, the incidence of *E. coli* bacteremia was highest in the under one year and over 56 year-old age groups. Previous population-based studies have documented the association of all bloodstream infections with old age (Hyle et al., 2005; Johnson et al., 2006). Uslan et al., 2007 identified an increased risk of *E. coli* bacteremia in females across all age ranges which contrasts the finding of an increase risk in only those above 55-year-old of age. In contrast, Kang et al., 2013 found that elderly males were at highest risk. The study showed that, solid tumors (19.2%), hematological malignancy (14.1%) and end-stage renal disease/post renal transplant (12.8%), are the most common underlying diseases and were identified as significant risk factors for health-care associated *E. coli* bacteremia.

Comparably, Kang et al., 2013 has found that, solid tumors, diabetes mellitus and liver diseases were the most common underlying diseases and predisposing factors for community onset bacteremia caused by ESBL producing *E. coli*. In a case controlled study from Spain of

96 patients with nosocomial blood stream infections (BSI) due to ESBL producing *E. coli*, the risk factors were found to be organ transplant, previous use of oxyimino-β-lactams, unknown BSI source and duration of hospital stay (Rodrguez-Bano et al., 2008). In addition, a population-based surveillance involving a total of 2368 episodes of *E. coli* bacteremia conducted in the Calgary Health Region has found that, the very young and the elderly were at highest risk for *E. coli* bacteremia. Additionally, dialysis, solid organ transplantation and neoplastic disease were identified to be the most important risk factors for acquiring *E. coli* bacteremia (Laupland et al., 2008). Among the 422 patients with neoplastic disease, 270 (64%) had malignant tumors, 96 (23%) had hematological malignancies, one patient had both a tumor and a hematological malignancy, and 55 (13%) patients had neoplastic disease in remission.

In another study by Chen et al., 2010, on the epidemiology of bloodstream infections in patients with haematological malignancies with and without neutropenia, the authors found that *E. coli* (12%) predominated the Gram-negative isolates causing BSI in neutropenic patients (Chen et al., 2010). Over the past two decades, treatment of *E. coli* bacteremia has become increasingly complicated by the emergence of antimicrobial-resistant *E. coli* strains, particularly those strains possessing acquired resistance genes encoding extended-spectrum beta-lactamases (ESBLs) and carbapenemases. Bloodstream infections with these resistant organisms have been associated with adverse clinical consequences and significant therapeutic challenge to treating physicians. The initiation of an antimicrobial agent is usually empirical, requiring knowledge of the likely pathogen and usual antimicrobial susceptibility patterns. This work has highlighted concerning trends towards greater antimicrobial resistance in *E. coli* causing bacteremia. However, in this study, both community-acquired and hospital-acquired *E. coli* isolates showed the highest sensitivity to carbepenem and amikacin followed by gentamicin and tazocin. The community-acquired isolates are more susceptible to amikacin, tazocin and ciprofloxacin than the hospital-acquired isolates. Similar to this study, Khanfar et al., 2009 found in his study, none of the strains isolated were resistant to carbapenems. In addition, recent studies showed that previous use of oxyimino-β-lactams or fluoroquinolones is a risk factor for ESBL-producing isolates in patients with bacteremia caused by E. *coli* (Quirante et al., 2011; Rodrguez-Bano et al., 2010). A retrospective cohort analysis (Rodriguez-Bano et al., 2006) has shown that, when compared with β-lactam/β-lactamase-inhibitor and carbapenem-based regimens, empirical therapy of ESBL-producing *E. coli* bacteremia with cephalosporins or fluoroquinolones were associated with a higher mortality rate. Resistance to drugs other than penicillins and cephalosporins was associated with increased mortality (Rodrguez-Bano et al., 2010). The mortality rate in this study (25%) is higher than previously (11.4 %) reported [6]. In a recent prospective cohort studies, carried out in hospitals from 31 countries that participated in the European Antimicrobial Resistance Surveillance System (EARSS), excess mortality associated with BSIs caused by MRSA and third-generation cephalosporin-resistant *E. coli* (G3CREC) is significant, and the prolongation of hospital stay imposes a considerable burden on health care systems.

These studies are essential to assist with the challenges of empiric antibiotic prescribed for those presenting to hospitals with suspected sepsis. As both community-acquired and hospital-acquired *E coli* isolates showed the highest sensitivity to imipenem, meropenem, in this study, it is believed in view of their excellent *in vitro* activity, carbapenems along with amikacin should be the initial empiric choice for serious life threatening infections caused by ESBL producing *Enterobacteriaceae*, with prompt de-escalation when culture and susceptibility results become available. In this study, there was no significant difference in the mortality rate between community and nosocomial bacteremia. Identification of risk factors for MDR organisms in patients presenting from the community with sepsis is necessary to help optimize patient outcomes and minimize the use of broad-spectrum antibiotics. To the authors' knowledge, this is the first report presenting data differentiating between nosocomial and community acquired ESBL *E. coli* bacteremia in Saudi Arabia. Continued surveillance, appropriate use of antibiotics and implementation of strict infection control measures are recommended to reduce ESBL frequency.

Conflict of interests

The authors declare that they have no conflict of interests.

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Abbreviations

CA, Community acquired; **HA,** hospital acquired; **ESBL,** extended spectrum beta lactamase; **ESRD,** end stage renal disease.

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Full Length Research Paper

A systematic review of antibiotic-resistant *Escherichia coli* and *Salmonella* data obtained from Tanzanian healthcare settings (2004-2014)

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Antibiotic-resistant *Escherichia coli* and *Salmonella* are an increasing challenge to global health. In Tanzania reliable data is limited for trends of resistance in major hospital-acquired pathogens. Data on the prevalence of antibiotic-resistant *E. coli* and *Salmonella* from Tanzanian sources (2004-2014) was extracted from PubMed and Google Scholar databases (April-June, 2015). Descriptive statistics and logistic-regression analysis were used to estimate the prevalence and trends for resistant *E. coli* and *Salmonell*a to selected antibiotics using R software. A total of 24 articles were availablefor review, of which 21/24 (87.5%) and 7/24 (29.2%) reported the prevalence of antibiotic-resistant *E. coli* and *Salmonella*, respectively. Across all studies the average prevalence of resistance to ampicillin and co-trimoxazole was higher for *E. coli* (81.6 and 77.7%, respectively) than for *Salmonella* (64.7and 59.3%, respectively). Both groups of pathogens were also resistant to ciprofloxacin (20-22%) and 3rd-generation cephalosporins (2.5-27.8%). A logistic-regression model for published data (2004-2014) indicated that during this period of time there has been a significant increase to amoxicillin/clavulanate, ceftazidime, ciprofloxacin and gentamicin in *E. coli* (*P*< 0.001), and a significant increase in resistance to ampicillin for *Salmonella* (*P* < 0.05).Decreased *E. coli* and *Salmonella* susceptibility to critical antibiotics threatens the effective treatment of these infections in Tanzania. Proactive strategies are needed to preserve these antibiotics that remain largely active against bacterial pathogens in Tanzania.

Key words: Antibiotic resistance, trends, nosocomial *E. coli*, *Salmonella*, Tanzania.

INTRODUCTION

Antibiotic resistance (AMR) is one of the major global- health challenges of the 21st Century (Huttner et al.,

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2013). Bacteria that are resistant to ≥ 3 antibiotic classes are conventionally referred to as “multidrug-resistant” andsuch microbes challenge existing treatment regimens for bacterial infections (Laxminarayan and Heymann, 2012; Thu et al., 2012). Multidrug-resistant bacteria often cause chronic diseases in people leading to long-term hospitalization, high morbidity and mortality (Feasey et al., 2012). *Escherichia coli* and *Salmonella* sp. (*S. enterica* subspecies *enterica*) commonly cause septicemic infections in Africa (Feasey et al., 2012; Anago et al., 2015). Multidrug-resistant *E. coli* and *Salmonella* often express extended spectrum beta-lactamases (ESBLs) that favour increased resistance to broad-spectrum beta-lactam antibiotics. These genetically encoded traits are usually located on plasmids that are transferable between bacterial strains and species (Sweta Gupta et al., 2013; Anago et al., 2015). Data on antibiotic resistance for pathogens is generally limited in sub-Saharan Africa (Leopold et al., 2014). In Tanzania, a situational analysis report by Global Antibiotic Resistance Partnership Working Group (GARP) called for a coordinated response to AMR problem and reveals baseline data for presence of antibiotic-resistant *E. coli* and *Salmonella* sp.in nosocomial infections (GARP-Tanzania, 2015), but there is no systematic mechanism for tracking trends in major hospital-acquired pathogens (WHO, 2014). This review focused on the prevalence and trends of antibiotic resistance for nosocomial *E. coli* and *Salmonella* as reported in the literature between 2004 and 2014. The analysis focused on antibiotics that are considered critical to Tanzanian healthcare settings by the WHO- Advisory Group on Integrated Surveillance of Antibiotic Resistance (AGISAR, 2011).

According to AGISAR, a critically important antibiotic (CIA) is the sole, or only one of limited available therapies to treat serious human disease such as pneumonia. Antibiotics are also considered critical when they are important for treating diseases caused by either (1) organisms that may be transmitted to people from non-human sources or, (2) human diseases caused by organisms that may acquire resistance genes from non-human sources. Antibiotics that meet criterion 1 or criterion 2 are referred to as highly important antibiotics (HIA) (WHO-AGSAR, 2011).

Results from this review focus on these important antibiotics with the goal of improvingtreatment guidelines for hospital-acquired infections and address the need for enhanced antibiotic stewardship strategies in Tanzania.

METHODS

Article search strategy and selection criteria

Search words “resistance” or “antibiotic resistance” or “multidrug resistance” and/or “*Salmonella* “or “*Escherichia*”, or “antibiotic susceptibility”, or “antibiotics”, or “antibiotic” or “bacteraemia” or “bacteriuria” and *Tanzania* were used with PubMed and Google Scholar electronic databases. Boolean operators, proximity search and mapping techniques (Boell and Cecez-Kecmanovic., 2010); Boell and Cecez-Kecmanovic, 2014) were employed to identify relevant articles. All articles published between 2004 and early 2015 that reported prevalence of antibiotic-resistant*E. coli* and *Salmonella* isolates from Tanzanian clinical specimens in healthcare settings were retrieved and analysed if antibiotic resistance data was reported based on Kirby-Bauer disc diffusion assays.

Statistical analysis

Extracted data were entered into a spreadsheet (Excel 2013, Microsoft Corp., Redmond, WA, USA). Tables and descriptive statistics were used to summarize data. Average prevalence (Number of resistant/total number isolates tested) for a 10-year period (2004-20014)and the proportion of antibiotic-resistant *E. coli* and *Salmonell*a [number of resistant/(number of sensitive +number of resistant isolates)]was computed for each antibiotic across all studies. Logistic-regression was used to assess trends in resistance for *E.coli* and *Salmonell*a to selected antibiotics for the data, published between 2004and 2014, using R software (v3.2.5, stats package). All results at $P< 0.05$ were considered statistically significant.

RESULTS

Description of search results

A total of 1,136 articles was retrieved and screened from PubMed (n=616) and Google Scholar (n=520) electronic databases between April and June, 2015 (Figure 1). Twenty-four articles (n=24) passed inclusion criteria set for this review (Table 1). The majority of the articles (16/24; 67%) consisted of cross-sectional, hospital-based studies (Table 1).

A hospital-based infection was defined as(1) an infection that was acquired by neonates within 10 days of birth in a hospital, or (2) inpatients showing symptoms of new infection >48 h following admission, or (3) community-acquired infections involving septicemic infection with the growth of pathogenic bacteria in a blood-culture that was obtained within the first 48 h of admission. Only one study by Blomberg et al.(2007), examined data from both community- and hospital-based infections. For cross-sectional studies the presence of bacterial pathogens (exposure) and antibiotic resistant infections (disease) were determined at the same point in time in a given population and the prevalence of exposure and/or diseases was assessed. A few studies (8/24; 33%) were either retrospective or prospective cohort studies (Table 1).

For retrospective studies bacteria were isolated from a cohort of individuals prior to the onset of the study and assessed for antibiotic resistance.prospective studies are rare in the field of antibiotic resistance, but typically involve a cohort of individuals that are identified and examined for the presence ofantibiotic-resistant bacteria relative to risk factors for carriage of antibiotic-resistant strains during a defined study period (Euser et al., 2009).

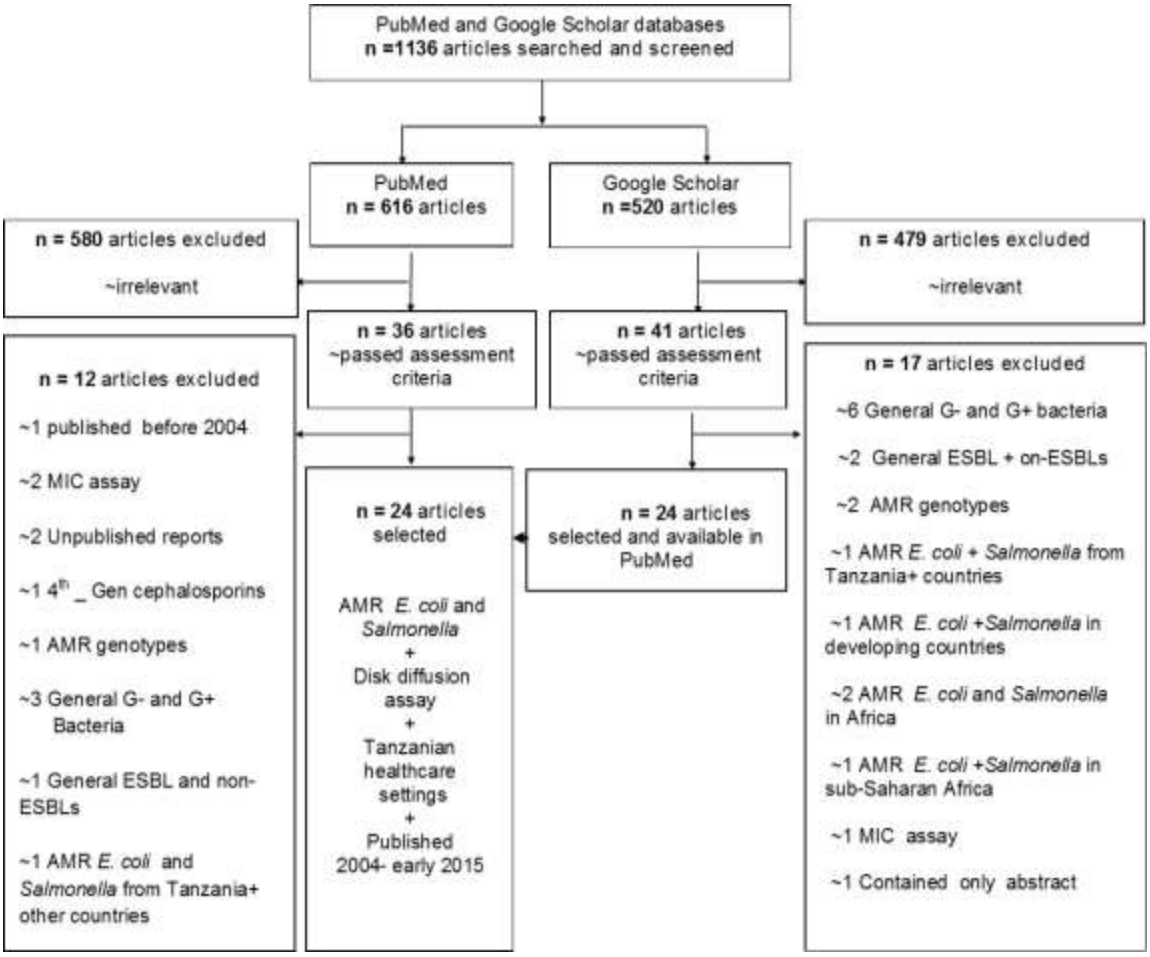

Figure 1. Flow diagram indicating an inclusion assessment of selected articles for systematic review.

Description of microbiological analysis

In the studies considered, clinical samples were collected directly from patients of varying age (neonates; 0-26 days; children; >5 and >18 years; adults, >65 years) and from stored samples (bio-bank). Blood was the main clinical type of sample (13/24; 54.2%) (Blomberg et al., 2004, 2007); Ndugulile et al., 2005; Mshana et al., 2009; Kayange et al., 2010; Moyo et al., 2010; Crump et al., 2011; Meremo et al., 2012; Mhada et al., 2012; Msaki et al., 2012; Christopher et al., 2013; Mushi et al., 2014). Other samples included urine (9/24; 37.5%), pus (6/24; 25%), and other body fluids (6/24; 25%. All studies employed disc diffusion assays for antibiotic susceptibility testing and *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were commonly used as quality control organisms. For selected studies, the susceptibility assays for critically important antibiotics (CIA) included ampicillin, amoxicillin/clavulanate, ciprofloxacin, ceftazidime, gentamicin, and meropenem and for highly important antibiotics (HIA), the assays were performed to co- trimoxazole, chloramphenicol and tetracycline.

Prevalence of antibiotic-resistant *E. coli*

In the last two decades there has been an increasing number of reports about antibiotic-resistant *E. coli* isolates from tertiary hospitals. In the selected studies, antibiotic-resistant *E. coli* from septicaemia (BSI) and urinary tract infections (UTI) was reported in seventeen studies (17/24; 70.8%), while four studies (4/24; 16.7%) reported other *E. coli*-associated infections such as surgical site infections (SSI) and diarrhoea (Table 1). When data were pooled from the 21 published reports, *E. coli* indicated high resistance to ampicillin (81.6%), tetracycline (74.9%) and co-trimoxazole (77.7%) (Table 2).

Prevalence of antibiotic-resistant *Salmonella*

Non-typhoidal *Salmonella enteric* serovar typhimurium

Table 1. Synopsis of studies included in systematic review (n=24 articles).

Study design	Healthcare setting[#]	Source of infection	Study population	Patient (n)	Reference
Cross-sectional	HLH	UTI	Resistant *E. coli*	5153	Blomberg et al. (2005) [1]
Prospective-cross-sectional	MNH	BSI	Resistant *S. tyhi, S. enteritidis, S. tyhimurium, S. newport*	1787	Blomberg et al. (2007) [2]
Cross-sectional	MNH	Bacterial infection	Resistant *E. coli*	7617	Blomberg et al. (2004) [3]
Prospective-cross-sectional	BMC	BSI	Resistant *E. coli Salmonella* spp.	634	Christopher et al. (2013) [4]
Prospective-cross-sectional	KCMC	BSI	Resistant *S. typhi*	403	Crump et al. (2011) [5]
Cross-sectional	MNH	UTI	Resistant *E. coli*	382	Fredrick et al. (2013) [6]
Cross-sectional	MNH	UTI	Resistant *E. coli Salmonella* Typhi	300	Lyamuya et al. (2011) [7]
Cross-sectional	MNH	SSI	Multidrug resistant *E. coli*	100	Manyahi et al. (2014) [8]
Cross-sectional	BMC	BSI	Resistant *E. coli*	945	Marwa et al. (2015) [9]
Cross-sectional	BMC	UTI	Resistant *E. coli*	247	Masinde et al. (2009) [10]
Prospective-cross-sectional	BMC	BSI	Resistant *E. coli Salmonella* sp.	346	Meremo et al. (2012) [11]
Cross-sectional	MNH	BSI	Resistant *E. coli*	330	Mhada et al. (2012) [12]
Cross-sectional	BMC	Lower limb ulcer	Multidrug resistant *E. coli*	300	Moremi et al. (2014) [13]
Retrospective	MNH	BSI	Resistant *E.coli Salmonella* sp.	13,886	Moyo et al. (2010) [14]
Cross-sectional	MNH	Diarrhoea	Resistant *S.* typhi, S. typhimurium, Enteritidis	280	Moyo et al. (2011) [15]
Prospective-cross-sectional	BMC	SSI	Resistant *E. coli*	250	Mawalla et al. (2011) [16]
Prospective cohort	KCMC	BSI	Resistant *E. coli*	181	Morpeth et al. (2008) [17]
Cross-sectional	MHC	UTI	Resistant *E. coli*	231	Msaki et al. (2012) [18]
Cross-sectional	BMC	Hospital infections	Multidrug resistant *E. coli*	800	Mshana et al. (2009) [19]
Prospective-cross-sectional	BMC	BSI	Resistant *E. coli*	770	Kayange et al. (2010) [20]
Cross-sectional	MNH	UTI	Multidrug resistant *E. coli*	50	Ndugulile et al. (2005) [21]
Cross-sectional	BMC	Hospital infections	Multidrug resistant *E. coli*	227	Mushi et al. (2014) [22]
Cross-sectional	MRH	Diarrhoea	Resistant *E. coli* 0157	275	Raji at al. (2008) [23]
Cross-sectional	BMC	UTI	Resistant *E. coli*	370	Festo et al. (2011) [24]

[#]HLH, Hydom Lutheran Hospital; MNH, Muhimbili National Hospital; BMC, Bugando Medical Center; KCMC, Kilimanjaro Christian Medical Center; MRH, Morogoro Regional Hospital; MHC, Makongoro, Health Center.

Table 2. Summary of antibiotic resistance prevalence among *Escherichia coli* in healthcare settings, Tanzania (2004-2014); (Ref: [1, 3, 4, 6 - 14, 16 - 24] (21 studies)).

Antibiotic [a] (*N)[b]	Prevalence range (%) of resistant *E. coli* in various studies	Average prevalence n[c]/*N (%)
Ampicillin[13] (2073)	53 - 100	1692/2073 (81.6)
Amoxicillin/clavulanate[10](1572)	38 - 100	551/1572 (35.1)
Cefotaxime[5] (151)	5 - 92	42/151 (27.8)
Ceftazidime[6] (1403)	5 - 50	161/1403 (11.5)
Tetracycline[10] (1570)	59 - 100	1177/1570 (74.9)
Gentamicin[14] (2098)	8 - 92	313/2098 (14.9)
Co-trimoxazole[18] (1881)	50 - 100	1462/1881 (77.7)
Chloramphenical[8] (407)	45 - 100	210/407 (51.6)
Ciprofloxacin[11] (899)	5 - 46	181/899 (20)
Nitrofuratoin[7] (1674)	4 - 32	350/1674 (20.9)
Meropenem[3] (271)	5 - 19	53/271 (19.6)

[a] superscripts (3-18) indicate the number of reviewed studies; [b] *N=Total number of tested *E. coli* isolates; [c] n= number of antibiotic-resistant isolates.

has emerged as a predominant cause of invasive bacterial infection among African HIV-infected individuals

Table 3. Summary of antibiotic resistance prevalence among *Salmonella* sp., in healthcare settings, Tanzania (2004-2014) (Ref: [2, 4, 5, 7, 11, 14, 15], (7 studies))

Antibiotic [a] (*N)[b]	Prevalence range (%) of resistant *Salmonella* in various studies	Average prevalence n[c]/*N (%)
Ampicillin[6] (136)	41 - 100	88/136 (64.7)
Amoxicillin/clavulanate[5] (67)	0 - 100	27/67 (40.3)
cefotaxime[2] (46)	0 - 3	1/46 (2.2)
Ceftazidime[2] (40)	0 - 3	1/40 (2.5)
Tetracycline[4] (59)	0 - 42	17/59 (28.8)
Gentamicin[4] (44)	0 - 29	7/44 (15.9)
Co-trimoxazole[7] (108)	0 - 100	64/108 (59.3)
Chloramphenical[4] (100)	21 - 85	29/100 (29)
Ciprofloxacin[4] (18)	0 - 100	4/18 (22.2)
Nitrofuratoin[2] (10)	0 - 20	2/10 (20)

[a] superscripts (2-7) indicate the number of reviewed studies; [b] *N=Total number of tested *Salmonella* isolates; [c] n= number of antibiotic-resistant isolates. For Meropenem no resistant *Salmonella* was detected.

and malnourished children with case fatality rates of 20 to 25% (Crump et al., 2011; Feasey et al., 2012). In sub-Saharan Africa, presence of invasive non-typhoidal *Salmonella* (NTS) has been reported by several authors (Mshana, Matee and Rweyemamu, 2013; Carden et al., 2015). Antibiotic-resistant *Salmonella* data from Tanzanian sources was reported in seven studies (7/24; 29%; Table1).*S. enterica* subsp *enteric* serovars Typhi, Typhimurium, Enteritidis and Newport were reported in four studies (4/7; 57.1%) (Blomberg et al., 2007); Crump et al., 2011; Lyamuya et al., 2011); Moyo et al., 2011). Three studies (3/7; 42.1%) reported *Salmonella* at the genus level (Moyo et al., 2010; Meremo et al., 2012; Christopher et al., 2013). Pooling data across studies demonstrated relatively high average resistance to ampicillin (64.7%) and co-trimoxazole(59.3%) (Table 3).

Prevalence of extended spectrum beta-lactamase producers (ESBLs)

Multidrug-resistant *Escherichia coli* and *Salmonella* that produce ESBLs are increasingly reported worldwide (Rogers, Sidjabat and Paterson, 2011; Manyahi et al., 2014; Rezai et al., 2015). For Tanzania occurrence of globally distributed *E. coli* ST 131 clone with β-lactamase and fluoroquinolone resistance was first reported in 2011 (Mshana et al., 2011). For this review ESBL producing *E. coli* were reported in five studies (5/24; 20.8%) (Ndugulile et al., 2005); Mshana et al., 2009; Manyahi et al., 2014; Moremi et al., 2014; Mushi et al., 2014). Pooling data across studies indicated that the average prevalence of ESBL producing *E. coli* in Tanzania was 39.2%. In these studies ESBL producing strains were frequently resistant to co-trimoxazole (76.9-92%), gentamicin (30.8-93%) and ciprofloxacin (45-92%).

ESBL genes ($bla_{\text{CTX-M-15}}$, $bla_{\text{SHV-12 and}}$ $bla_{\text{OXA\_48}}$) were identified in *E. coli* by PCR for two studies (Ndugulile et al., 2005; Mushi et al., 2014).

Trends of antibiotic resistance

Between 2004 and 2014 there was asignificantly increasing trend ($P< 0.001$) for *E. coli* resistance to critically important antibiotics [amoxicillin/clavulanate (38 to 100%);ceftazidime (5 to 50%); ciprofloxacin (5 to 46%) and gentamicin (8 to 92%)] and an insignificant change for ampicillin resistance (53 to 100%;$P>0.05$). For highly important antibiotics [co-trimoxazole (50 to 100%) and tetracycline (59 to 100%)], the trend of increasing resistancewas insignificant ($P> 0.05$)(Table 4). For *Salmonella*the trend of increasing resistance was significant ($P < 0.05$) for ampicillin (41 to 100%) but no significant trend was detected for co-trimoxazole, amoxicillin/clavulanate, ceftazidime, ciprofloxacin, gentamicin and tetracycline (Table5).

DISCUSSION

Published data (2004-2014) aboutthe prevalence of antibiotic-resistant *E. coli* and *Salmonella* from hospital-acquired infections in Tanzania suggests thatthere was a high average prevalence of resistanceto ampicillin (81.6 versus 64.7%, *E. coli* and *Salmonella*, respectively) and co-trimoxazole (77.7 versus 59.3%) (Tables 2 and 3). Comparable results were reported for *E. coli* in Kenya (ampicillin, 95%; co-trimoxazole, 95%) (Sang et al., 2012), Ethiopia (ampicillin, 100%; co-trimoxazole, 62.9%) (Beyene and Tsegaye, 2011; Kibret and Abera, 2011), Zimbabwe (ampicillin, 84.5%; co-trimoxazole, 68.5%) (Mbanga et al., 2010) Ghana (ampicillin; 66.7%; co-trimoxazole, 68.2) (Hackman et al., 2014), Nigeria

Table 4. The odds ratio of antibiotic-resistant *Escherichia coli* in healthcare settings, Tanzania (2004-2014)[a].

Antibiotic [b]	Odds ratio	95% Confidence interval
Critically important		
Ampicillin	1.08[ns]	0.90 -1.28
Amoxacillin/clavulanate	2.13***[c]	1.86 - 2.45
Ceftazidime	0.61***	0.49 - 0.76
Ciprofloxacin	0.59***	0.49 - 0.70
Gentamicin	0.73 **	0.61 - 0.88
Highly important		
Co-trimoxazole	1.12[ns]	0.95 - 1.32
Tetracycline	1.09[ns]	0.89 - 1.31

[a] Odds ratios as estimated by logistic-regression analysis. Chloramphenicol, Nitrofuratoin and Meropenem were not analysed due to insufficient data; [b] Categories according to WHO advisory group on integrated surveillance of antibiotic resistance (AGISAR, 2011); [c] * $P < 0.05$ ** $P < 0.01$;*** $P < 0.001$; ns, non-significant ($P > 0.05$).

Table 5. The odds ratio of antibiotic-resistant *Salmonella* sp., in healthcare settings, Tanzania (2004-2014)[a].

Antibiotic[b]	Odds ratio	95% Confidence interval
Critically important		
Ampicillin	1.77*[c]	1.16 - 2.68
Amoxicillin/clavulanate	1.00[ns]	0.70 - 1.43
Ceftazidime	1.00[ns]	0.61 - 1.68
Ciprofloxacin	1.24[ns]	0.79 - 1.96
Gentamicin	1.13[ns]	0.71 - 1.78
Highly important		
Co-trimoxazole	1.49[ns]	0.45 - 5.51
Tetracycline	0.20[ns]	0.02 - 1.39

[a] Odds ratios as estimated by logistic-regression analysis. Chloramphenicol, Nitrofuratoin and Meropenem were not analysed due to insufficient dat; [b] Categories according to WHO advisory group on integrated surveillance of antibiotic resistance (AGISAR, 2011); [c] * $P < 0.05$; ns, non-significant ($P > 0.05$).

(ampicillin, 100%; co-trimoxazole 75.6%) (Yah et al., 2007) and South India (ampicillin, 99%; co-trimoxazole, 68.7%) (Razak and Gurushantappa, 2012). High resistance to ampicillin and co-trimoxazole is a challenge for treatment of bacterial infections in Tanzania where ampicillin is used as an empirical therapy and co-trimoxazole is used as a prophylaxis to prevent opportunistic infections among HIV-infected individuals (Hamel et al., 2008; Marwa et al., 2015).The use of robust and affordable diagnostic tools for bacterial infections in Tanzanian hospitals in accordance is highly recommended to restrict ineffectiveadministration of these antibiotics. Resistance to these “older” antibiotics is particularly unfortunate because alternatives will be increasingly expensive in a country that can ill-afford increased medical expenses.

Relatively high resistance of *E. coli* and *Salmonella* to critical antibiotics such as ciprofloxacin (20 versus 22.2%, respectively) was evident (Table 2 and 3).Over the course of the review period, there was a statistically significant increase in *E. coli* resistance to amoxacillin/clavulanate ($P < 0.001$), ceftazidime ($P < 0.001$), ciprofloxacin ($P < 0.001$) and gentamicin ($P < 0.01$), whereas no significant trend was observed for *Salmonella* (Table 4 and 5). This disparity in trends suggests that there is a greater need to scrutinize treatment decisions for *E.coli* infections. Reduced susceptibility of nosocomial *E. coli* pathogens to critical antibiotics was also reported by others in Nigeria (ciprofloxacin, 15.4%) and Iran (ciprofloxacin, 16.8%) (Khameneh and Afshar, 2009; Akinkunmi et al., 2014). Conversely, the rapid spread of ciprofloxacin resistance in a widely disseminated *S.typhi* strain (haplotype H58) both in Africa and Southeast Asia (Berkley et al., 2001; Chiou et al., 2014) alerts for the possible emergence of

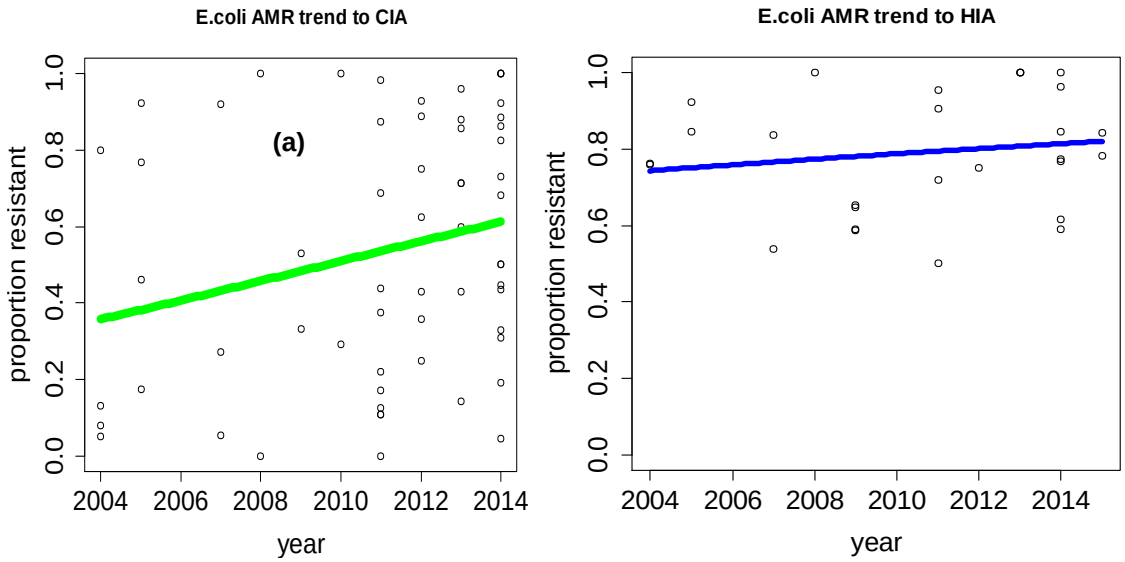

Figure 2. a: Trend in antibiotic resistance of *Escherichia coli* to critically important antibiotics (CIA, ampicillin; gentamicin; amoxicillin/clavulanate; ciprofloxacin and ceftazidime) in 10 year period (2004-2014), healthcare settings, Tanzania; b: Trend in antibiotic resistance of *E. coli* to highly important antibiotics (HIA, co-trimozazole and tetracycline) in 10 year period (2004-2014), healthcare settings, Tanzania.

this lineage of bacteria in Tanzania, particularly if there is heavy reliance of fluoroquinolones to treat Typhoid infections.

Detection of ESBL and carbapenamase-producing strains among *E.coli* isolates has been reported in several studies (Ndugulile et al., 2005; Moremi et al., 2014; Mushi et al., 2014). These isolates were highly resistant to amoxicillin/clavulanate (88.5-90.9%), ceftazidime (50-100%), ciprofloxacin (45.5-61.3%) and gentamicin (72.7-93.5%).Emerging resistance to extended beta-lactams and fluoroquinolones is an escalating public health concern for the management of infections among children and immuno-compromised individuals. Dissemination of ESBL strains in healthcare settings has been previously reported in various countries, including Kenya (Kiiru et al., 2012), Benin (Anago et al., 2015), Iran (Rezai et al., 2015), Brazil (Ferreira et al., 2011) and Bangladesh (Lina et al., 2014). Access to diagnostic tools that can detect ESBLs in local healthcare settings needs to be enhanced. In Tanzania,like many developing countries, laboratory capacity to confirm ESBL phenotypes is limited and diagnostic tools for infections are commonly unavailable or unreliable (Berkley et al., (2001).

Findings from community-acquired infections were addressed by Blomberg et al. (2007). Unexpectedly, they found that *E.coli* and *Salmonella* pathogens were more prevalent in confirmed community cases compared with hospital-acquired cases (32.9 and 17.9%, respectively). Furthermore, it was evident that *E. coli* pathogens were more susceptible ($P< 0.05$) to amoxicillin-clavulanate (75% vs 31%), cefuroxime (88% vs 54%), ceftazidime (88% vs 46%) and cefotaxime (88% vs 50%) in community-acquired infections compared with hospital-acquired cases, respectively. These results are consistent with reports from other community-level studies conducted in South Africa (McKay and Bamford, 2015), Iran (Hashemi et al., 2013), France (De Bus et al., 2013) and Spain (Junquera et al., 2005).

There is evidence of increasing numbers of *E. coli* and *Salmonella* resistance to critical antibiotics in Tanzania over the past 10 years (Figures 2a and 3a). This is probably explained in part by a high prevalence of nosocomial infections and growing rates of hospitalization reported in developing countries. This increased service demand has likely increased reliance on more potent antibiotics as initial or empirical treatment because they act against a wide range of pathogens (Laxminarayan and Heymann, 2012; Thu et al., 2012). As a consequence, this practice facilitates selection and persistence of bacterial strains resistant to critical antibiotics (Mshana et al., 2009; Meremo et al., 2012). High resistance to these antibiotics in nosocomial *E. coli* and *Salmonella* infections has been reported in Cameroon (Lonchel et al., 2012), India (GARP-India, 2011) and Latin America (Salles et al.,2013).Decreased *Salmonella* non-susceptibility to highly important antibiotics (Figure 3b) may suggest an increased proportion of susceptible isolates to this group of antibiotics for the period between 2004 and 2014. Nevertheless, a relatively high resistance (59.3%) to co-trimoxazole may be explained by its common usage as an alternative treatment for infectious diarrhoea (Casburn-Jones and Farthing, 2004) (Table 3). In

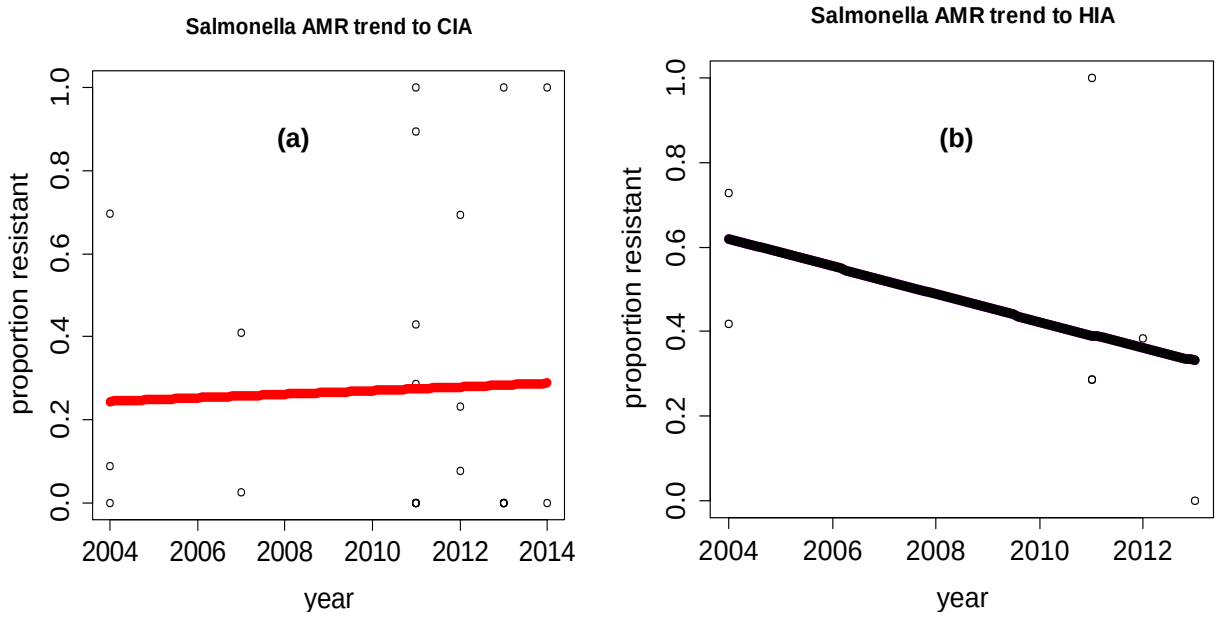

Figure 3. a: Trend in antibiotic resistance of *Salmonella* to critically important antibiotics (CIA, ampicillin; gentamicin; amoxicillin/clavulanate; ciprofloxacin and ceftazidime) in 10 year period (2004-2014), healthcare settings, Tanzania; b: Trend in antibiotic resistance of *Salmonella* to highly important antibiotics (HIA, co-trimozazole and tetracycline) in 10 year period (2004-2014), healthcare settings, Tanzania.

contrast, high *Salmonella* susceptibility to highly important antibiotics such as co-trimoxazole, has been reported in various countries like Nepal (1995-2015: co-trimoxazole, 98.8%) (Shrestha et al., 2016), Southern India (2009-2011:co-trimoxazole,95%) (Choudhary et al., 2013), and Montenegro (2005-2010: co-trimoxazole, 96.3%) (Mijovic, 2012).These findings suggest that local susceptibility testing of highly important antibiotics may be essential for timely treatment of *Salmonella* infections in low-income populations like Tanzania where access and/or options tomore potent antibiotics is generally limited (Laxminarayan et al., 2015).

It is important to note that the majority of the reviewed studies relied on data from hospital-acquired infections. Only one study included data from community- acquired infections and consequently, it is possible that the numbers reported in the literature are upwardly biased. This can happen when patients self-medicate prior to presentation at a hospital and this probably increases the possibility of isolating resistant strains. The use of Clinical and Laboratory Standard Institute (CLSI) guidelines was reported only by a subset of studies. Thus, the accuracy of any susceptibility data from studies that employed different guidelines might have caused variation in results. Finally, the lack of ESBL phenotype data in many studies might result in an underestimate of the prevalence of multidrug-resistant bacteria. Overall, high *E. coli* and *Salmonella*non-susceptibility to ampicillin and co-trimoxazole suggests that these antibiotics can be inappropriate empirical treatment for major nosocomial infections in Tanzania. Further, decreased *E. coli* and *Salmonella* susceptibility to amoxicillin/clavulanate, ceftazidime, ciprofloxacin and gentamicin threatens the effective treatment ofthese infections in Tanzania. Implementing proactive strategies in antibiotic stewardship to preserve the effectiveness of critical antibioticsthat appear to remain largely effective against bacterial pathogensin Tanzania is crucial. Applying enhanced infection control measures would limit further spread of resistant bacteria in healthcare settings and community as well.

Conflict of Interest

The authors declare that there is no conflict of interest.

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Bacterial populations of mosquito breeding habitats in relation to maize pollen in Asendabo, south western Ethiopia

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Mosquito larvae feed on particulate organic matter including microorganisms. This study was conducted to investigate the diversity and abundant bacteria of *Anopheles* mosquito larva breeding habitats and to evaluate the contribution of maize pollen as source of nutrients for bacterial growth. The nutrient composition (COD, NH_3-N and TP) of the larva breeding habitats water samples were measured by HACH 2010. Bacteria were isolated and enumerated from 18 water samples of larva habitats. The abundance of bacteria in the larva breeding habitats was significantly different. Bacteria were found to be grown abundant in habitats close to maize pollen sources (tasseled zone). This implies that maize pollen contributes to bacterial abundance. The Pearson Correlation showed that there was positive relationship between bacterial abundance and physicochemical characteristics of the water samples. The bacterial population in the habitat was dominated by species of *Bacillus*, *Pseudomonas*, *Micrococcus* and *Serratia*. The dominant bacteria were tested for their capability to grow on maize pollen medium. The growth kinetics of bacteria on maize pollen broth was performed to 18 h culture using JENWAY spectrophotometer at 600 nm wave length. The bacteria could show optimum growth on Maize Pollen broth at 15 g/l as of nutrient broth. The release of maize pollen during anthesis in rainy season in habitats close to larva breeding pool and its nutriment quality support proliferation of large array of bacteria which results in increased larval nourishment. Increased malaria transmission in Asendabo could thus be caused as the bacteria serve as source of nutrients for mosquito larva.

Key words: Maize pollen, microbial flora, mosquito larva habitat, maize pollen broth, bacterial abundance.

INTRODUCTION

Mosquitoes are vectors of pathogenic protozoan, viruses, and nematodes which cause the disease malaria, yellow fever, and lymphatic filariasis respectively to humans (Okogun et al., 2003; Maekawa et al., 2011) to humans and related diseases in domestic animals. Anopheles are medically important mosquito, transmit malaria disease. The genus Anopheles comprises of 421 species, which are distributed in the world (Kettle, 1995). They breed in

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many types of temporary water bodies, but differ in preferences to particular types of water body (Mwangangi et al., 2007; Machault et al., 2009).

Anopheles gambiae is one of the most important vectors of malaria and that is widely distributed throughout Afro tropical region. It breeds in small, sun ex-posed, fresh water pools which are often free of vegetation (Muirhead-Thomson, 1958). The larvae of *Anopheles gambiae* are also found at the coast in intertidal, salt water swamps (Ketlle, 1995).

The larval stages of *A. gambiae* complex are frequently found in transient water bodies, where suspended solid particles are abundant to provide turbidity. The other Anopheline species such as *Anopheles quadrimaculatus* develop in relatively clear water by exploiting bacteria-rich surface environment of permanent marshes through their interfacial feeding strategy (Yemane et al., 2000).

Bacteria (Cyanobacteria) are widely distributed in mosquito breeding habitats and used as food for mosquito larvae. The size of adult mosquito population is largely dependent on the presence of plankton (Mala and Irungu, 2011), the nutritional role of organic matter, and microbial fauna in the mosquito larval habitats (Okech et al., 2007). Larval food consists of a variety of living and non-living materials, such as dust, bacteria (Cyanobacteria), unicellular algae (zoo flagellates) and filamentous algae, fungi and small metazoans (rotifers and crustaceans), spores and in-sect scales. Bacteria are the most important microbial constituents of mosquito larvae food and the mosquito can grow on culture made only of bacteria (Merritt et al., 1992).

The amount of organic matter and nutrient in the water is important factors in temporary small water bodies of the habitats of many Anopheline mosquitoes. In these shallow habitats there is a close association between the organic matter in the water and the organic content of the underlying soil (Muirhead-Thomson, 1958).

Maize (*Zea mays L*., Poaceae) is the most important cereal in the world after wheat and rice with regard to cultivation areas and total production (Abdulrahaman and Kolawole, 2006). The name maize is derived from the South American Indian Arawak-Carib word *mahiz*. It was introduced into Ethiopia probably in the 16th century by the Portuguese (Phillip, 1995).

Maize is the second most important cereal crop grown by Ethiopian farmers (Adugna and Melaku, 2001), with major production regions located in the southern, western, southwestern, and eastern high lands and used predominantly for human consumption in the country (Getahun et al., 2000). It is estimated that the crop covers 1.3 m ha of cultivated land. Maize is used predominantly as human food consumption in the country (Adugna and Melaku, 2001). In general, the area coverage of maize is high in the Oromia region followed by SNNPR and lower in Amhara region (Berhanu et al., 2007). Maize pollen provides important sources of nutrients for adult and larvae of *An. arabiensis* in Ethiopia (Yemane et al., 2003). The mosquito adult developed more in maize pollen supplemented medium than algal supplemented medium (Yemane et al., 2000). Maize pollen contains various diffusible, water-soluble as well as non-soluble proteins and other nutrients. The nutrient analysis of BH660 Hybrid maize pollen contains 19% protein, 48% carbohydrate, 2% fat, 2% ash and 9% moisture contents Bezawit Eshetu (2007). This maize plant releases its pollen and provides more nutrients to the development of mosquito larvae (Yemane et al., 2003b).

While the microbial biomass associated with different larva aquatic habitats is well documented, the taxonomic composition of these microbes and their response to common environmental stressors is poorly understood (Muturi et al., 2013).

The goal of this research were to study the microbial constituents of *Anopheles* mosquito larva breeding habitats and evaluate the nutriment contribution of maize pollen to the growth of bacteria, and to investigate the correlation of the larva breeding water micronutrient to the abundance of bacterial isolates.

The hypotheses tested were that maize pollen support different quantities of abundant taxonomic groups of bacteria and exposure of microorganisms to maize pollen source can alter bacterial abundance in mosquito aquatic habitats and its contribution to malaria transmission.

MATERIALS AND METHODS

The nutrient contents of larva breeding water samples were measured and correlated with the abundance of bacteria and hence to the proliferation of mosquito larva. The abundant bacteria were isolated, characterized and identified, and the bacterial abundance was correlated to the availability of maize pollen.

Sample collection

Water samples were collected from eighteen different study sites in Asendabo, Waktola Asendabo (longitude: 37.23", latitude: 7.73" and altitude: 1870 m). Triplicate water samples were collected by using sterile jar, vials, glass bottles and sterile plastic bag. Soon after collection the samples were transported to AAU for microbiological studies and investigations. The maize pollen was collected from Arbaminch, Mirab Abaya (irrigation fed area) (Latitude: 06°18', longitude: 37°47'), where maize shed the pollen lately. The pollen collected was kept in sterile glass bottle at room temperature until the time of its evaluation as a nutrient source for microbial growth.

Habitat characterization

Sample sites were described in terms of vegetation, physical water condition, exposure of the site to sunlight (Herrel et al., 2001; Barros et al., 2011; Abebe Animut et al., 2012; Afrane et al., 2012). The study sampling sites were divided in to three zones with regard to the availability of maize pollen source: tasseled zone was a zone with its male maize fluorescence remains attached and pollen was allowed to pollinate by natural process (wind), the detasseled zone was a site from where the male fluorescence was removed and

shaken to pollinate and the buffer zone was where the pollen source was of a different form other than maize pollen (taro, teff and green pepper).

Isolation of bacteria from mosquito larva habitats

One milliliter of each water sample was diluted with 9 ml of sterile distilled water in screw caped test tubes up to 10^{-5} series of dilution. From first to four series of dilution, 0.1 ml aliquots of water sample was spread in to nutrient agar plates and incubated for 48 h at 25°C. Nutrient agar (Oxoid) contains (g/l): 'Lam-Lemco' powder 1.0, Yeast extract 2.0, Peptone 5.0, Sodium Chloride 5.0 and Agar 15.0. Each colony was observed on a plate and checked for its purity and the impure colony was transferred to nutrient broth and streaked into nutrient agar plates to re- isolate and purify the bacteria. Following growth, bacterial colonies were further purified and preserved on nutrient agar slants at 4°C for further studies (Fry and Zia, 1982). The total counts of bacterial isolates were enumerated using the formula (number of CFU = number of colonies x Dilution factor of the plate counted). The bacterial isolates were designated as AS6B, AS6C, AS11A, AS12B, AS15B, AS16D, AS17D, AS17E, AS19B and AS20C.

Identification of bacterial isolates

The bacteria isolates were identified by using cultural, morphological, biochemical and physiological characteristics according to Bergey's Manual of Systematic Bacteriology (1984).

Cultural characteristics of bacterial isolates

The cultural characteristics of bacterial isolates such as colony color, colony elevation, colony margin and colony surface were inspected visually in a plate (Aneja, 2005).

Morphological characteristics of bacterial isolates

Morphological characteristics of bacterial isolates such as spore stain, gram staining, shape and motility tests were determined by light microscopy (Bisen and Verma, 1994; Aneja, 2005).

Biochemical characteristics of bacterial isolates

Biochemical characteristics of isolates, such as Oxidation-Fermentation (OF) test (Bisen and Verma, 1994), Catalase activity (Chester, 1979), urea hydrolysis test, SIM test (Aneja, 2003), MR-VP test, oxidase test and citrate utilization (Bisen and Verma, 1994), carbohydrate fermentation test (Aneja, 2003), hydrolysis of gelatin (Bisen and Verma, 1994; Aneja, 2003), and starch hydrolysis (Aneja, 2003; Mondal et al., 2015) were characterized.

Physiological characteristics of bacterial isolates

For the physiological tests the bacterial isolates were streak inoculated in to nutrient agar medium. The bacterial isolates were inoculated in to the medium with different pH (5, 7, and 10). For salt tolerance tested the bacterial isolates were inoculated into the medium with different salt concentration (2% NaCl, 5% NaCl, 7% NaCl, 10% NaCl and 12% NaCl). The temperature optima was tested by inoculating the isolates at 4, 10, 15c, 40, 45, 50, 55 and 65°C (Aneja, 2003; Bisen and Verma, 1994; Mondal et al., 2015).

Measurement of chemical parameters of water samples

The determination of chemical concentration of the water samples were carried out.

Measurement of ammonia nitrogen

The ammonia nitrogen concentration was measured by Nesslers method. The spectrophotometer was programmed at 380 with wavelength 425 nm. Twenty five ml of water sample was filled in to a 30 ml glass vial (sample cell). To this sample, 1 ml of Nessslers reagent was added, mixed by shaking and allowed to stand for one minute to complete the re-action. All the samples were analyzed for ammonia nitrogen species by taking the reading at HACH 2010 spectrophotometer methods (APHA, 1998).

Measurement of total phosphorous

Total phosphorus (TP) was determined by acid per-sulfate digestion method (APHA, 1998). Five milliliter water sample was added in to total acid hydrolyzable test vial. Potassium per sulfate powder pillow was added and dissolved by shaking. Then, the sample was digested for 30 min at 150°C on COD reactor. After digestion, the digested samples were allowed to cool at room temperature. Two ml of 1.54 normal NAOH was added, mixed and the outside of the tube cleaned with a towel. From this point each sample was treated at a time, zero each vial in HACH 2010 spectrophotometer. One phosver 3-reagent powder pillow was added, shaken for 10 to 15 s. The timer was shift for 2 min; the outside of tube was cleaned with towel and read by using HACH 2010 spectrophotometer (APHA, 1998).

Measurement of COD

The COD reactor was adjusted to 150°C .Two milliliter sample was added to COD vial tube plus one blank. The content of vials was mixed by inverting the vials. The outside of the vial was cleaned with towel and digested for two hours. After two hours of sample digestion the reactor was turned off, the samples were removed from the reactor and allowed to cool at room temperature. The digested cool samples were read on HACH 2010 spectro-photometer programmed at 435 of wave length 620 nm (APHA, 1998).

Evaluation of growth of bacterial isolates in maize pollen

Maize pollen collection and processing

Maize pollen was collected by using hard cover post during anthesis of maize crop. The pollen was put and allowed to dry in open air. The dried pollen powder was shed by hand crushing from the pollen stalk, sieved and kept for future use as media.

Maize pollen broth media preparation

5, 10 and 15 g maize pollen powder was boiled in distilled water (1 L) to dissolve the nutrient contents. After boiling the preparation was allowed to settle. The supernatant decanted from the precipitate was sterilized at 121°C 15 lb pressure for 15 min. The pH of pollen medium was adjusted to 7.2 for bacterial isolate growth evaluation before sterilizing. For Maize pollen agar media preparation, 15 g agar was added to the same preparation as before on another test and was used for physical (visual) evaluation of bacterial growth.

Table 1. Abundance of bacteria from water samples presented in terms of log mean colony forming units per ml (log Mcfu/ml) with standard error, isolated from larva habitats.

Sample site	Bacteria
1	4.52±0.18
2	5.10±0.67
3	5.62±0.012
4	5.53±0.35
6	6.24±0.32
7	6.18±0.39
9	4.71±0.21
10	5.33±0.31
11	5.56±0.23
12	6.12±0.24
14	4.39±0.39
15	5.99±0.35
16	5.60±0.12
17	4.77±0.94
18	4.50±0.24
19	5.92±0.11
20	4.79±0.52
22	5.34±0.11

One-way ANOVA showed that at α=0.05 there was significant difference in bacteria ($F_{(17, 36)} = 2.48$, $P=0.011$) population densities in water samples from the larvae breeding habitats.

Growth kinetics evaluation

The growth of microorganism on a maize pollen broth media was evaluated by inoculating the isolates on a medium prepared solely of maize pollen. From 18 h culture, 10^7 number of cells per ml was inoculated into the pollen broth medium (contain 5, 10 and 15 g/l). The optical density (OD) reading was recorded from zero hours of inoculation up to 8 h of incubation within one hour intervals using JENWAY spectrophotometer at 600 nm wave length. The growth of bacterial isolates on maize pollen agar (Pollen in g/l: 10, 15, 20, 25, 30 and 35) was also observed by streak inoculation. Additionally, the optical density reading of bacterial isolates was also measured in nutrient broth medium for comparison. OD reading was taken for the blank made of only maize and nutrient broth at con-centration of 5l, 10l and 15 g/l used as a control.

Data analysis

All the data generated was analyzed using SPSS version 20. One way ANOVA was computed for analyzing the variation of microbial abundance and habitat water chemistry. The Pearson correlation analysis was used to analyze the correlation between physicochemical and biological parameters of the water samples collected from larva habitats. The total count of bacteria (CFU) were determined by standard formula (CFU = Number of colonies × DF of sample plated).

RESULTS

Sample site description

Sample sites were covered by vegetation including algae, grasses. Water condition was clear, turbid, flowing and/or standing. Some of the site exposed directly to sunlight, partially shaded or shaded.

The sites were borrow pit formed by people when they dug to extract mud for their house construction, puddles and the hoof prints on cattle way and human foot prints. They were divided in to three zones with regard to the availability of maize pollen source: Tasseled zone was the zone where male maize fluorescence remains attached and pollen was allowed to pollinate by natural process (wind); the detasseled zone was the site where the male maize fluorescence was removed and pollinated by hand and the buffer zone was where the pollen source was different from maize pollen such as taro, teff and green pepper.

Isolation of bacteria from water samples of larva habitats

Twenty different bacterial colonies were isolated from eighteen water samples of larva breeding habitats. The ten selected bacterial isolates for further studies in terms of their abundance was designated as AS6B, AS6C, AS11A, AS12B, AS15B, AS16D, AS17D, AS17E, AS19B and AS20 C. The colonies isolated were white, yellow, red, opaque, cream and orange colored. The white colonies were rough surface with leafy margin, the cream colonies were mucoid, and the other colonies were smooth with entire margin. The designation of isolates was the same as for yeast isolate designation.

Enumeration of bacteria from water samples of larva breeding habitats

Bacteria was found to be abundant in water from site number 6 (6.24±0.32) followed by site 7 (6.18±0.39) and site 12 (6.12±0.24) (Table 4). Site 14 contained the least number of bacteria (4.39±0.39) (Table 1).

Evaluation of bacterial growth on maize pollen medium

Growth of bacterial isolates on maize pollen agar (PA)

Among the ten most abundant bacteria selected from the isolates of water samples of the larva breeding sites, the bacterial isolate AS15B, AS17D, AS17E, AS19B and AS20C generally showed more growth on maize pollen agar than the other isolates (AS6B, AS6C, AS11A, AS12B and AS16D). As few as 5 g of maize pollen per liter was found to be enough to support the minimum growth of bacterial isolates.

Evaluation of bacterial isolates on maize pollen broth

The minimum growth of bacterial isolates was observed

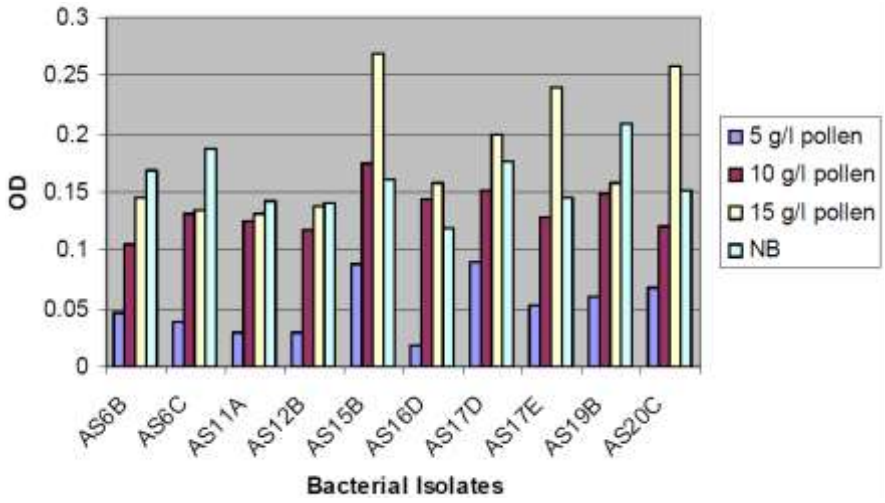

Figure 1. Schematic presentation showing bacteria growth on maize pollen and nutrient broth. NB, Nutrient broth; OD, optical density.

at 5 g/l of maize pollen (Figure 1). At concentration of 10 g/l of maize pollen, the bacteria showed appreciable growth. At 15 g/l of maize pollen concentration, the growth observed for isolates AS6B, AS6C, AS11A, AS12B and AS16D were almost similar but lower than the growth observed by the isolates AS15B, AS17D, AS17E, AS19B and AS20C. From the mean value of OD measured, isolates AS15B, AS17D, AS17E, AS19B and AS20 showed even better growth at 15 g/l of maize pollen concentration than in nutrient broth.

Measurement of nutrient contents of water samples from larva habitats (mean ± SEM)

The COD concentration measured from the water ample showed that it was not significantly different between the sample sites ($p>0.05$) at $\alpha=0.05$. Sample site 10 showed the highest COD (247.67+103.22) concentration. Sample site 6 and 9 were the second in concentration of COD (200+83.34). Sample site 14 showed the least concentration of COD (11.0+4.58).

There was no significance difference between the amounts of ammonia nitrogen concentration measured from the water sample ($P>0.05$, $\alpha=0.05$). The highest NH_3-N was measured from water sample 9 (7.62±3.22) (Table 2) and the lowest was measured from water sample 16 (0.77±0.21). The highest NH_4^+ concentration was measured from water sample 9 (9.81±4.27) and the lowest from water sample (0.99±0.27).

The concentration of reactive soluble phosphate-phosphorous (SRP-P) or PO_4^{3-} was the highest in water sample 2 (5.0±2.05) and the lowest in water sample 1 (0.79±0.33). The concentration of Total phosphorous (TP) was measured the highest from water sample 2 and 10 (1.63±0.68) and the least from water sample 1 (0.27±0.11)

Correlation of biological and physicochemical parameters

Pearson's correlations were calculated to test for relationships between bacterial abundance and environmental variables for bacterial communities in the larval breeding pool. The results indicated that bacterial abundance was positively but weakly correlated with COD ($r^2=0.096$). The concentration of total phosphorous in a breeding site was positively but weakly correlated with the abundance of bacteria ($r^2=0.179$). On the other hand the abundance of bacteria was negatively and weakly correlated with NH_4^+ ($r^2= -0.064$) and NH_3-N ($r^2= -0.063$) (Table 3).

Identification of bacterial isolates

Based on the abundance in water samples ten bacterial isolates were selected for studies. The bacteria were identified to the genus level on the basis of their cultural and morphological (Table 3), biochemical (Table 4) and physiological characteristics.

Cultural and morphological characterization of bacterial isolates

Ten bacterial isolates were selected in terms of their abundance in the water samples. The ten bacterial isolates produced white, cream, yellow, red, silver (opaque) and orange colony color on nutrient agar medium (Table 4). The colony surface was smooth,

Table 2. Nutrient concentration of water samples from larva habitats.

Site no. sample	COD (mg/l)	Total-P (mg/l)			NH_3-N (mg/l)		
		PO_4^{3-}-P	P_2O_5	TP	NH_3-N	NH_4^+	NH_3
1	110.67±46.09	0.79±0.33	0.58±0.24	0.27±0.11	4.01±2.31	5.15±2.96	4.87±2.80
2	174.0±72.53	5.0±2.05	3.9±1.63	1.63±0.68	5.87±2.57	7.56±3.31	7.15±3.13
3	126.33±52.65	2.42±1.01	1.79±0.75	0.79±0.33	1.57±0.28	2.02±0.36	1.9±0.34
4	158.33±66.01	3.32±1.38	2.48±1.03	1.10±0.46	7.55±3.03	9.73±3.90	9.19±3.67
6	200±83.34	2.37±0.99	1.74±0.72	0.79±0.33	2.08±0.01	2.67±0.12	2.53±0.11
7	105.67±44.14	1.58±0.66	1.16±0.48	0.54±0.22	1.94±1.09	2.50±1.40	2.35±1.32
9	200±83.34	2.95±1.23	2.21±0.92	0.95±0.39	7.62±3.22	9.81±4.27	9.27±4.03
10	247.67±103.22	4.95±2.06	3.69±1.54	1.63±0.68	2.59±0.63	3.33±0.80	3.12±0.74
11	94.67±39.43	1.74±0.72	1.26±0.53	0.58±0.24	5.20±2.72	6.67±3.49	6.29±3.30
12	58.0±24.13	4.63±1.93	3.48±1.45	1.53±0.64	1.78±0.32	2.27±0.40	2.15±0.38
14	11.0±4.58	1.42±0.59	1.05±0.44	0.49±0.21	3.49±0.55	4.48±0.68	4.25±0.66
15	196±77.0	3.27±1.36	2.48±1.03	1.05±0.44	3.49±1.91	4.49±2.46	4.23±2.32
16	47.33±19.78	1.85±0.77	1.37±0.57	0.58±0.24	0.77±0.21	0.99±0.27	0.94±0.26
17	94.67±39.43	1.90±0.79	1.42±0.59	0.63±0.26	3.91±2.04	5.05±2.63	4.77±2.49
18	158.00±65.87	2.84±1.18	2.11±0.88	0.95±0.39	1.34±0.77	1.72±0.99	1.64±0.95
19	79.0±32.87	3.32±1.38	2.48±1.03	1.11±0.46	2.89±0.73	3.89±0.92	3.51±0.88
20	126.33±52.65	1.47±0.61	1.10±0.46	0.48±0.2	1.34±0.57	1.74±0.73	1.64±0.69
22	168.33±70.12	3.27±1.36	2.42±1.01	1.05±0.44	2.08±0.55	2.68±0.72	2.56±0.69

Data presented in Mean ± SEM.

Table 3. Correlation of biological and physicochemical characteristics of water samples.

	AB
AB	1
COD	0.096
NH_3-N	-0.063
NH_4^+	-0.064
NH_3	-0.065
PO_4^{3-}	0.178
P_2O_5	0.173
TP	0.179

AB = Abundance of bacteria

rough and mucoid (Table 4). The gram reaction and KOH test showed that 5 isolates were gram positive, rod shaped, 4 isolates were gram negative, rod shaped and one isolate was gram positive coccus. The endo spore stain showed that 5 isolates formed central and ellipsoidal endospore (Table 4).

Biochemical characterization of bacterial isolates

Seven isolates showed positive test for gelatin liquefaction and the remaining three showed negative result (Table 5). For starch hydrolysis test 4 isolates showed positive and six isolates showed negative result.

Carbohydrate fermentation test showed that none of the isolates fermented lactose, 7 isolates produced acid from glucose with no gas and 5 isolates produced acid from sucrose with no gas (Table 5). All the ten isolates neither produced H_2S nor indole in SIM medium. Five isolates showed positive test result for MR and four isolate showed positive test result for VP test (Table 5). All isolates showed positive test for catalase and oxidase test. None of the isolates used citrate as the sole carbon source for growth. Only one isolate showed positive test result for urease test result. Seven isolate showed positive motility test result in SIM medium (Table 5).

Physiological characterization of bacterial isolates

The optimum temperature for all bacterial isolates was between 20 and 40°C. Only two out of ten isolates rough and mucoid (Table 4). The gram reaction and KOH rough and mucoid (Table 4). The gram reaction and KOH showed growth at 4°C (AS16D and AS20C), 50 and 55°C (AS17E and AS20). All isolates were capable of growing at 2% NaCl in nutrient agar medium. None of the isolates showed growth at 12% NaCl concentration. Six isolates showed growth at 5% NaCl, three isolates grown at 7% NaCl and two isolates showed growth at 10% NaCl in nutrient agar solution. The optimum salt tolerance for the isolate was variable. The optimum pH for the isolate was 7 to 10 in which all isolates showed growth. Only three

Table 4. Cultural and morphological characterization of bacterial isolates of water samples from larval habitats.

Bacterial isolate	Colony color	Colony texture	Gram rxn	Shape	Endospore	Motility
AS6B	Orange	Smooth	+	Coccus	N	_
AS6C	Reddish	Smooth	_	Rod	N	+
AS11A	Yellow	Smooth	_	Rod	N	+
AS12B	Yellow	Smooth	_	Rod	N	+
AS15B	White	Rough	+	Rod	E, C	+
AS16D	Silver (Opaque)	Smooth	_	Rod	N	+
AS17D	White	Rough	+	Rod	E, C	+
AS17E	Cream	Mucoid	+	Rod	E,C	_
AS19B	White	Rough	+	Rod	E,C	+
AS20C	Cream	Mucoid	+	Rod	E,C	_

N, Do not form endospore; E, ellipsoidal; C, Central.

Table 5. Biochemical characterization of bacterial isolates from water samples of larval habitats.

Characteristics	AS6B	AS6C	AS11A	AS12B	AS15B	AS16D	AS17D	AS17E	AS19B	AS20C
Gelatin liquefaction	-	+	-	-	+	+	+	+	+	+
Starch hydrolysis	-	-	-	-	+	-	-	+	+	+
Lactose fermentation	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Glucose fermentation	NA	NA	A	A	A	NA	A	A	A	A
Sucrose fermentation	NA	NA	NA	NA	A	NA	A	A	A	A
H_2S production	-	-	-	-	-	-	-	-	-	-
Indole test	-	-	-	-	-	-	-	-	-	-
MR-rxn	-	+	-	-	+	+	+	-	+	-
VP-rxn	-	+	_	_	_	+	-	+	-	+
Citrate utilization	-	-	-	-	-	-	-	-	-	-
Urease test	-	-	-	-	+	-	-	-	-	-
Catalase test	+	+	+	+	+	+	+	+	+	+
Oxidase test	+	+	+	+	+	+	+	+	+	+

A, Produce acid; NA, acid not produced; +, showed positive test; -, showed negative test.

isolate showed growth at pH 5.

Eventually consulting the Bergey's Manual of Systematic Bacteriology confirmed that the bacterial isolates were identified as *Bacillus*, *Micrococcus*, *Pseudomonas* and *Serratia* species (Table 6).

DISCUSSION

Mosquito larval habitat ecology is important in determining larval densities and species assemblage. This in turn influences malaria transmission in malaria endemic area. Understanding larval habitat ecology is, therefore, important in designing malaria control program. Understanding larval habitat characteristics in terms of environmental attributes and identifying relationships between biotic and abiotic factors are important for developing novel methods of vector control in communities with a high propensity to harbor *Anopheles* mosquitoes. In this study the microbial ecology of larval habitats in South Western Ethiopia, Asendabo and factors that influence Anopheles larval densities and diversity (Mwangangi et al., 2007) was investigated. This microbiological study of Anopheline larva habitats was sought to understand the ecologies of mosquito larvae and microbial species composition in the mosquito larva habitat. In this study, the composition and diversity of mosquito larva habitat associated bacteria were investigated. The dominant bacteria isolates in the larva habitats were identified to the genus *Bacillus*, *Pseudomonas*, *Micrococcus* and *Serratia*.

The immature stages of anopheles are non-selective filter-feeders of organic particles suspended in the water suspension and of microorganisms such as bacteria, yeasts, protozoans and fungi (Pereira et al., 2009). Hence, the bacteria identified from the larva natural habitat may be constituent of the larva potential diet. Anopheline larvae ingest particles in their aquatic habitat

Table 6. Physiological characterization of bacterial isolates from water samples of larval habitats.

Bacterial isolates	pH optima			Salt tolerance (% NaCl)				Temperature optima (°C)	Identified to
	5	7	10	2	5	7	10		
AS6B	+	+	+	+	-	-	-	20-40	*Micrococcus*
AS6C	-	+	+	+	-	-	-	10-40	*Serratia*
AS11A	-	+	+	+	+	-	-	20-40	*Pseudomonas*
AS12B	-	+	+	+	**+**	-	-	20-40	*Pseudomonas*
AS15B	+	+	+	+	**+**	+	-	20-40	*Bacillus*
AS16D	-	+	+	+	-	-	-	4-40	*Serratia*
AS17D	+	+	+	+	+	+	-	20-40	*Bacillus*
AS17E	**+**	+	+	+	-	-	**+**	20-55	*Bacillus*
AS19B	**+**	+	+	+	**+**	+	-	20-40	*Bacillus*
AS20C	**+**	+	+	+	+	-	**+**	4-55	*Bacillus*

AS, Asendabo; Numbers (6, 11, 12, 15, 16, 17, 19 and 20): Representative sample site from which bacteria isolated; Letters (A, B, C, D & E): Code given different isolates.

indiscriminately (Yemane, 2003) and therefore micro-organisms, which are in the natural habitat, may be the components of the larval diet that most determine the growth of larva (Wotton et al., 1997). *Bacillus*, *Serratia*, and *Pseudomonas* from our habitat isolates were similar to bacteria isolated from *Anopheles* mosquitoes by Tchioffo et al. (2013). These results indicate that the bacterial species are closely associated with *Anopheles* midguts may have come from larvae feeding. The present study revealed that the maize pollen alone, without any supplementary nutrient, was found to be a complete nutrient source, which allowed the bacteria to grow on. Bezawit Eshetu (2007) reported that nutrient analysis of BH660 hybrid maize pollen contains 19% protein, 48% carbohydrate, 2% fat, 2% ash and 9% moisture content. These provide the nutriment to the microorganism as well as to the Anopheline larvae.

Anopheline larvae usually feed on microorganisms (Muturi et al., 2013) and detritus (Merritt et al., 1992). The microorganisms are able to thrive on maize pollen, which in turn serve as Anopheline larvae nutriment. The maize pollen contribute to the larval nutriment directly as the mosquito larvae use water soluble nutrient content of pollen (Yemane et al., 2003) and indirectly support the larval diet by allowing the proliferation of micro-organisms to comprise the microlayer of water bodies.

The population densities of bacteria are larger in tasseled zone than the detasseled and buffered zone. This is, may be, due to the nutritional enrichment of habitat of tasseled zone by maize pollen, which supplies additional nutrient to the microorganisms. Maize pollen dispersed about 60 m by wind and land on the larval breeding site nearby (Yemane et al., 2000). The bacteria in sites close to maize benefited more from the pollen, proliferate and become abundant.

The concentration of nutrient in the water sample of larva habitat drained and accumulated from surface runoff fertilized and irrigated soil was low. The concentration of ammonia nitrogen was lower and this might be the result of the transfer of volatile and semi volatile compounds (ammonia) from water bodies to the atmosphere (Schindler, 2001). The amount of ammonium concentration was low as compared to finding report by Kudom (2015). The small amount of ammonium concentration in the habitat of larvae suggests that larval excretion of NH_4^+ was being balanced by its removal through microbial metabolism. However, without measurement of specific rates of ammonia oxidation, nitrification, and product consumption, it is difficult to assess the mechanism of larval impact on the process (Kaufman et al., 199). According to EEPA (2002), the standard for free ammonia is 5 g/l. In comparison to this EEPA (2002) standard, the concentrations of ammonia nitrogen is higher at sample site 2, 4 and 9 than the minimum standard and lower than the standard for the other study sites.

The concentration of SRP-P in the site was measured from 0.79 in site 1, which was the least concentration compared to 5.0 g/l in site 2 and 10 , which was the highest concentration measured in this study. This was smaller in comparison to the SRP-P measured from surface water (about 2-5 g/l) by Swift (1981) in marsh site, but higher from common types of *A. gambiae* breeding habitats (Imam and Deeni, 2014).

The relatively high levels of ammonia nitrogen relative to TP encountered in this microhabitat suggest phosphorous limitation (Kling et al., 2007). The smaller reactive soluble phosphate-phosphorous (RSP-P) in site 1 may have been the result of uptake by probably more abundant bacteria in the breeding pool. Because of its high reactivity, phosphorous may have lost due from the microhabitat by precipitation or adsorption on to inorganic particles which were continuously suspended as a result of mixing (Rediat, 2008).

The nutrient concentration of larval breeding habitat varies among the habitat; it can significantly influence the

development and body size of emerging *An. gambiae*. Larval development duration and adult body size decrease but pupation rate in-creases when the organic content of the soil substrate increases. This supports the relation between larval habitat quality and mosquito response in terms of development time and body size of adult mosquitoes. The soil type of mosquito aquatic habitat is critical for larval development (Pfaehler et al., 2006). Maize pollen releases its nutrient content in to the larvae breeding site, in which the microorganism thrive on and facilitate the nutrient geochemical recycling, which create conducive microhabitat for larvae to flourish.

Furthermore Garros et al. (2008) suggested that algae and microorganisms are the main food source of *A. quadrimaculatus* larvae, and mosquito larvae are not discriminatory in the type of food they ingest. Analysis of the biodiversity present in the larval habitats was carried out and this study suggests that there is wide range of microorganisms available in natural habitats. This study can be used to improve our understanding of the larval ecology of African malaria vectors and to facilitate the development of new mosquito control strategy through bringing the changes on the larva habitats.

Even though there was no statistically significant difference between the concentrations of ammonia nitrogen in the breeding site, the sites differed in the abundance and diversity of microorganism. This study results showed that bacterial abundance in fresh waters can be stimulated by inorganic nutrients (Le et al., 1994).This might be due to the supplementary nutriment of bacteria by the maize pollen, which had provided soluble nutritional material for microbes. Nutrient released by the decomposition of maize pollen is equally available and important for proliferation of bacteria and other microorganism as well. The water sample nutrient concentration in larval habitats of anopheline probably has crucial roles in the resource ecology of these mosquitoes (Mala and Irungu, 2011).

There were more bacteria in water sampling sites with pollen access (sample site 6, 7, 11, 12 and 15) than sites of low pollen access. This confirmed that maize pollen is an important nutrient to bacteria in a natural setting. Therefore, it requests additional investigation on using maize cultivar, which could with stand drought and produce crop in a dry season than during rainy season. This will minimize the availability of maize pollen to microorganism as well as to mosquito larvae and hence, results in vector control and reduction of malaria transmission.

The accumulations of organic matter under the surface film of water bodies form the surface micro-layers. Heterotrophic microorganisms benefited from this organic matter for growth, and they, in turn, are fed upon by Anopheles mosquito larvae. From laboratory experiments by Wotton *et al*., 1997 mosquito larvae, *An. gambiae* and *An. quadrimaculatus*, grow most rapidly where surface microlayers are present and, especially, where labile dissolved organic matter (may be contributed by the maize pollen shedding in our case) is added to promote growth of microorganisms, which are the components of the larval diet that most affect growth (Wotton et al., 1997).

Conclusion

The results of the present study have revealed that the chemical parameter of water samples from larva habitats of Anopheles mosquito is correlated to the abundance of microorganisms. The microbial flora of the water sample was dominated by the Bacteria species, *Bacillus*, *Micrococcus*, *Pseudomonas* and *Serratia*.

The abundance of bacteria in the breeding site is seen to be largely a function of maize pollen concentration. Bacteria, fungi and yeasts are the microflora which constitutes the feeding surface microlayer of Anopheline larva habitat in the natural habitat, from which the larva obtain their feed. The larvae in natural habitat feed indiscriminately on particulate organic matter and microorganisms and hence, the bacteria isolated and identified from the water samples of larva breeding habitats could also be good nutrient source for larva. The microorganism of larva breeding habitat, close to maize pollen source obtain more nutrient from this nutritious plant in addition to nutrient from substratum, become more abundant and enrich the water surface microlayer with microbial flora.

Recommendation

Based on this study the need to verify the types and numbers of microorganisms in the gut of Anopheles mosquito larvae in relation to their habitats (water bodies) is necessary.

Conflicts of Interests

The authors have not declared any conflict of interests.

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