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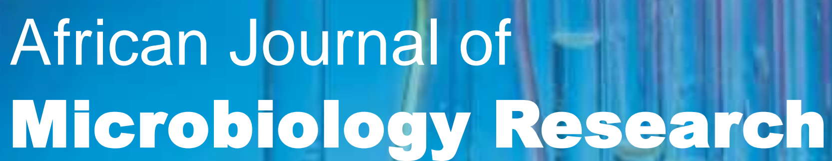

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African Journal of Microbiology Research

Review

Drug resistance in African animal trypanosomes: A review

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Animal African trypanosmiasis (AAT) is the most important factor contributing to the sub potential performance of livestock. AAT is caused by *Trypanosoma congolense*, *Trypanosoma vivax* and *Trypanosoma brucei*. Chemotherapy and chemoprophylaxis are the major means of combating the disease. An estimated 17 million cattle are treated with trypanocides annually. The emergence of drug-resistant trypanosome strains is considered a serious problem in trypanosomiasis control particularly for the resource-poor, at-risk populations and farmers in Africa. Trypanocidal drug resistance is the decreased or absence of sensitivity of trypanosome strains to standard quality trypanocidal drugs at the dose recommended in a good veterinary practice. Different resistance mechanisms are acquired independently through exposure to different drugs. A good example for this is cross resistance for diminazene and isometamidium. Trypanosomiasis drug resistance has been officially reported in 21 African countries. Moreover, certain African countries reported the presence of multi drug resistance. Safeguarding the available trypanocidal drugs is mandatory to reduce the devastating impact of the disease.

Key words: Resistance, trypanosome, African animal trypanosomiasis.

INTRODUCTION

African trypanosomes cause human African trypanosomiasis (HAT) and African animal trypanosomiasis (AAT), a debilitating disease of humans and domestic animals in the humid and sub-humid zones of Africa, respectively (Muhanguzi et al., 2015). Most African trypanosomes are transmitted by tsetse flies (Glossinidae), which inhabit in many parts of the continent (Kebede and Animur, 2009). AAT is the most important factor contributing to the sub potential performance of livestock (Chanie et al., 2013).

AAT is caused by *Trypanosoma congolense*, *Trypanosoma vivax* and *Trypanosoma brucei* species. *Trypanosoma evansi* causes 'Surra' in camels (*Camelus dromedarieus*) (Mbaya et al., 2010). Concurrent infections can occur with more than one species of trypanosome (IICAB, 2009). Even though the name of the disease is called as African trypanosmiasis, *T. vivax* and *T. evansi* by virtue of their transmission by

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haematophagous biting flies also occur in Asia, Central and North and Southern America (Hoare, 1966).

The occurrence of animal trypanosomiasis coincides with the distribution of tsetse fly vectors which includes the regions between latitudes 14°N and 29°S (OIE, 2013). AAT causes a great economic loss in the livestock industry with an estimated 3 million cattle death annually. Estimated direct production losses in cattle to African farmers are between 1 and 4 billion US dollars per year (Swallow, 2000; Abebe and Jobre, 1996).

Infection of cattle by one or more of the three species results in sub-acute, acute or chronic disease. The cardinal clinical sign observed in AAT is anemia, within a week of infection with *T. congolense* and *T. vivax*. Moreover, there is a pronounced decrease in packed cell volume (PCV), hemoglobin and red blood cell count. These manifestations are observed in goats experimentally infected with *T. vivax* (Osman et al., 2012).

Chemotherapy and chemoprophylaxis are the major means of combating the disease. An estimated 17 million cattle are treated with trypanocides annually (Kristjanson et al., 1999). However, effectiveness of these drugs is limited by factors such as parasite resistance (Afework et al., 2000; Melaku and Birrasa, 2013) and unacceptable toxicity (Onyekwelu, 1999). The emergence of drug-resistant trypanosome strains is considered a serious problem in trypanosomiasis control particularly for the resource-poor, at-risk populations and farmers in Africa (Kagira and Maina, 2007). The presence of indiscriminate drug utilization practices and subsequent complaints over the efficacy of the available trypanocidal drugs supplemented the presence of resistant strains (Fiarcloug, 1963).

TRYPANOCIDAL DRUG RESISTANCE

Drug resistance also called drug fastness (Uilenberg, 1998) is the heritable loss of sensitivity of a micro-organism to a drug to which it was sensitive (Sinyangwe et al., 2004). Similarly, trypanocidal drug resistance is defined as the decreased or absence of sensitivity of trypanosome strains to standard quality trypanocidal drugs at the dose recommended by the manufacturer and administered according to the good veterinary practice (Peregrine et al., 1996; Uilenberg, 1998). Information on the extent and significance of the problem of drug resistance is scant (Sinyangwe et al., 2004).

Over the last decades there has been a dramatic slowdown in the development of new antimicrobials, which increases the need to preserve existing antimicrobials (Grace, 2015). Due to this, only small group of chemoprophylactic and chemotherapeutic trypanocidal compounds are currently in use and new compounds are unlikely to be available (Barret, 2004). This is also the major limitation of trypanosomiasis treatment, because there is unwillingness of pharmaceutical companies to invest in development of drugs against trypanosomiasis (Geerts et al., 2010). Therefore, the exiting trypanocidal drug should be safeguarded. Moreover, it is clear that medicinal plants play a prominent role against various human and animal diseases. A variety of medicinal plants and plants extracts have been reported for their significant anti-trypanosomal activity (Assefa, 2017).

Practically, treatment is mainly carried out by the livestock owners themselves without any supervision by veterinary personnel (Geerts et al., 2010). Consequently, treatment given by livestock owners is not without serious drawbacks because most farmers do not have adequate knowledge on diagnosis and the appropriate drug to use even in areas of high prevalence of trypanosomiasis. Since trypanocides are frequently used in the absence of diagnosis or used to treat conditions of which they are not effective, emergency of resistance is apparent (Holmes et al., 2004).

When the trypanosome is resistant to more than one drug, it is considered as multidrug resistant. In this case, different resistance mechanisms are selected independently through exposure to different drugs. A good example for this is cross resistance for dimenazene and isometamidium (Black et al., 2001).

Different factors could affect the development of resistance against trypanocidal drugs; some of the major factors are as shown in Figure 1.

MECHANISMS AND GENETICS OF RESISTANCE TO TRYPANOCIDES

Diminazene

Diminazene was introduced onto the market as trypanocide for domestic livestock in 1955. But, in initial experiments, the compound was shown to be highly active against both trypanosome and *Babesia* species. It is the only trypanocidal drug used against *Babesia* spp. It is one of the most commonly used trypanocide drug (Geerts and Holmes, 1998). This might be due to its higher therapeutic index than other trypanocides in most livestock, low incidence of toxicity (Peregrine and Mamman, 1993) and being active against trypanosomes that are resistant to other trypanocides used in cattle (Williamson, 1970).

Even though it has low incidence of resistance, some trypanosomes might have innate resistance subsequent to the first usage of diminazene aceturate in the field. Population of *T. congolense* and *T.vivax* were described in Nigeria that appeared to be innately resistant to diminazene (Jones-Davies, 1968). Furthermore, Wiliamson (1960) suggested that west african popluation of *T. vivax* express a higher level of natural resistance to diminazene than *T. congolense.* However, whether such

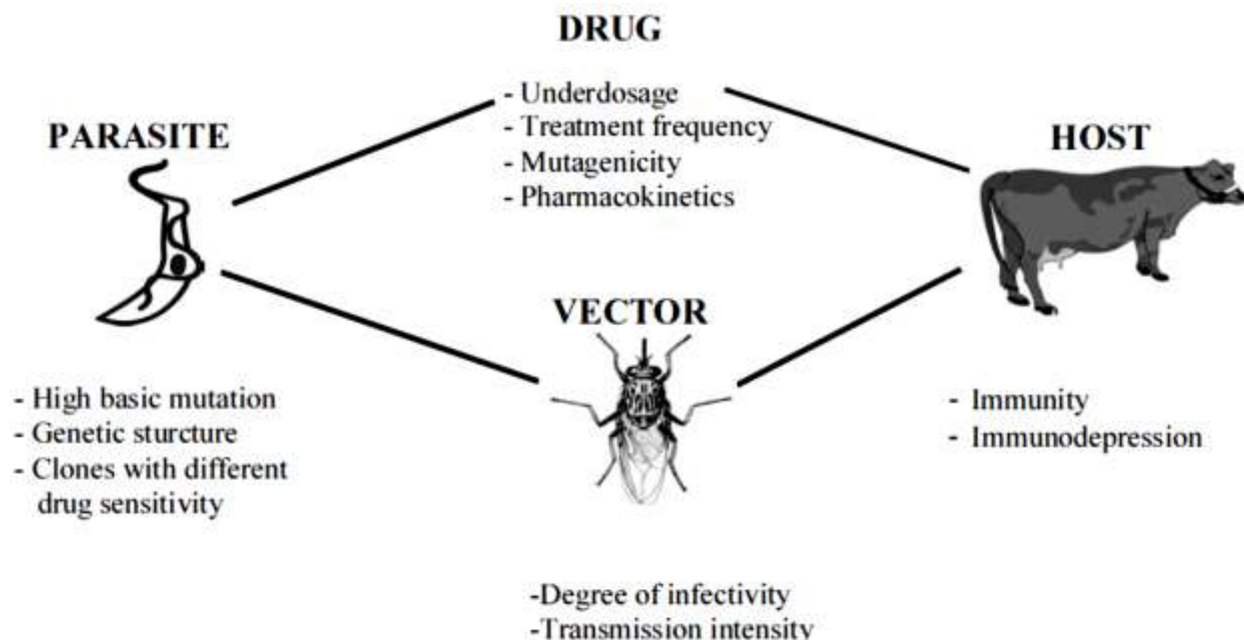

Figure 1. Major factors influencing the development of drug resistance (Geerts and Holmes, 1998).

resistance is a result of cross-resistnace induced by other drugs such as quinapyramine or natural is not clear (Gray and Roberts, 1971).

The accumulation of diminazene has been shown to be markedly reduced in arsenical-resistant *T. brucei*, *T. evansi* and *T. equiperdum* owing to alterations in the P_2-type purine transport system (Carter and Fairlamb, 1993; Carter et al., 1995).

It is also suspected that diminazene resistance is multi-factorial (Delespaux et al., 2006). The T*e*DR40 gene, which is a novel gene encoding protein that appeared to have a ubiquitous cellular localization (Witola et al., 2004), might be a contributing factor to resistance linked to the alteration of the gene coding for the P_2-type purine transporters. It is probable that diminazene resistance is the result of the cumulative effect of these two distinct resistance mechanisms (TeDR40 and P_2-type purine transporter) (Delespaux et al., 2006).

It has been urged that diminazene will not promote the development of resistant trypanosome strains even if sub-therapeutic doses are administered. But, resistance development as a result of the use of sub-therapeutic doses of diminazene and/or intensive use of the drug in the strain of *T. congolense* was reported. Further field studies confirmed the possibility of resistance in sub-therapeutic dose (Mbwambo et al., 1988).

Isometamidium

The amphiphilic cationic phenanthridine, isometamidium chloride, has been used in the field for several decades prophylactically or therapeutically for livestock suffering from trypanosomiasis. It is mainly used against infections with *T. congolense* and other *Trypanosoma* species (Leach and Roberts, 1981).

The first case of resistance to homidium and cross-resistance between homidium and isometamidium was reported in 1967 (Delespaux and De Koning, 2007). Decreased levels of drug accumulation have been observed in drug resistant populations of *T. congolense* (Sutherland et al., 1991). Experimental studies have demonstrated the occurrence of drug resistant trypanosomes to isometamidium (Schonefeld et al., 1987). This has been confirmed and reported by several authors (Codjia et al., 1993; Sinyangwe et al., 2004; Mulugeta et al., 1997).

The transport of isometamidium was known to be energy dependent, as it was reduced in the presence of metabolic inhibitors such as SHAM/glycerol (Sutherland and Holmes, 1993). In *T. brucei*, the P_2 adenosine transporter may be responsible for part of the isometamidium uptake as the drug inhibits P_2-mediated adenosine uptake (De Koning et al., 2005).

The mechanism of resistance to isometamidium chloride, however, is less clear despite certain suggested mechanisms from experimental finding results. Decreased levels of drug accumulation have been observed in drug resistant populations of *T. congolense* (Sutherland et al., 1991) and later work found indirect evidence of an increased efflux of drug from resistant trypanosomes (Sutherland and Holmes, 1993).

Resistance to isometamidium is mostly associated with cross-resistance to homidium (Peregrine et al., 1997),

and it could be speculated that these structurally related compounds might share the same uptake mechanism albeit that their distributions within the trypanosome are slightly different. Isometamidium is mainly concentrated in the kinetoplast, whereas homidium spread much more diffuse throughout the trypanosome (Boibessot et al., 2002).

Homidium

Homidium, which were introduced during 1950s, is active against *T. congolense* and *T. vivax* infections in cattle. Homidium salts are the main therapeutic drugs used in the management of clinical trypanosomiasis in animals (Holmes et al., 2004). It was widely used during the 1960s but due to the spread of resistance and its mutagenic activity, its use has greatly decreased (Holmes et al., 2004). The first report on the resistance of the drug was reported during 1960s (Jones-Davies, 1968).

The resistance mechanism to this drug is still unknown even if some studies suggested that it is similar to isometamidium (Shiferaw et al., 2015). Isometamidium differs from homidium by an additional moiety of *m*-amidinophenyl-*azo*-amine. The resistance mechanism thus seems to be specific for the homidium moiety, not the *m*-amidinophenyl-*azo*-amine functional group common to diminazene (Wragg et al., 1958).

Quinapyramine

Liao and Shen (2010) tried to look for physical changes, including proteins and genes, involved in the quinapyramine-resistance. Based on the confirmation made by other authors regarding the involvement of some enzymes in the production of drug-resistance in trypanosomes (Venegas and Solari, 1995; Iten et al., 1997), Liao and Shen (2010) detected for some isoenzymes. The enzymes analyzed were isoenzyme bands of hexokinase, glucose-6-phosphate dehydrogenase, alanine transaminase and aspartate aminotransferase, but they showed that the four isoenzymes were not involved in the quinapyramine resistance. And the soluble proteins of the 7 *T. evansi* lines was studied, their study implied that the two protein bands of 15.79 kDa in the lines, R1, R2, R4, R6 and R7, and 19.76 kDa in the three lines with higher levels of the antrycide resistance.

THE IMPACT OF RESISTANCE

The impact of drug resistant trypanosomes on livestock productivity has been assessed by fewer studies. But, it is imperative to assess not only the distribution of the problem, but also the constraints it encounters on effective control of the disease and economic impact (Melaku and Birrasa, 2013).

Increasing drug resistance increased trypanosome prevalence in cattle three-fold and in tsetse two-fold at a treatment frequency of once-a-month. The benefits of increased trypanocide treatment frequency diminish rapidly with increasing resistance (McDermott et al., 2000).

Previous study showed that trypanocidal drug resistance had no significant impact on the PCV and body weight losses of goats infected by *T. vivax* except for a few highly virulent strains. This conclusion contradicts the general believe that the development of resistance against trypanocides would leave farmers helpless in trypanosome infested areas (Vitouley et al., 2012).

A study to assess the impact of drug-resistant trypanosomes on the productivity of the local cattle was carried out by Codjia et al. (1993) in the Ghibe valley, Ethiopia. The area had a high prevalence of multiple drug resistance. In their study, the authors indicated that calf mortality was rather high, incidence of abortion was increased and the financial and economic returns were also affected.

DISTRIBUTION OF TRYPANOCIDAL DRUG RESISTANCE

The problem of drug resistance in animal trypanosomes is highly spreading geographically to many regions in which trypanosomiasis occurs (Grace et al., 2009; Geerts and Holmes, 1998). Decades after first case of drug resistance in trypanosomes, Clausen et al. (1988) confirmed multiple resistance trypanosomes isolates in the pastoral area of Samorogouan in Burkina Faso. Moreover, resistance developed by trypanosomes to trypanocidal drug, has been reported from East Africa (Mulugeta et al., 1997).

There is a report on a five-fold increase in the prevalence of dimenazene resistance over a seven year period in the Eastern Province of Zambia, suggesting, there might be a worsening of the problem. Trypanosomiasis drug resistance has been officially reported in 17 African countries (Burkina Faso, Chad, Ivory coast, Ethiopia, Kenya, Mali, Somalia, Sudan, Tanzania, Uganda, Zimbabwe, Zambia, Mozambique, Cameroon, Nigeria, Guinea, and Central African Republic) (Delespaux et al., 2008). But recently, this number is increased to 21 African countries (Tsegaye et al., 2015).

In addition, in 10 African countries including Ethiopia (Tsegaye et al., 2015), Sudan (Mohammed-Ahmed et al., 1992), Nigeria and Kenya, multiple drug resistance trypanosomes that decreased sensitivity to the common trypanocidal drugs (dimenazene, isometamidium and homidium) have been reported (Mulugeta et al., 1997;

Table 1. Report on drug resistant trypanosomes.

Country	Trypanosmes	Resistance to	References
Zambia	Tc	I	Sinyangwe et al. (2004)
	Tc	ID	Chitanga et al. (2011)
Mali	Tv	I	Mungube et al. (2012)
	Tc	ID	Mungube et al. (2012)
Burkina Faso	Tv	ID	Sow et al. (2012)
Mozambique	Tc	ID	Jamal et al. (2005)
Ethiopia	Tc	ID	Moti et al. (2012)
Zambia	Tc	I, D, ID	Sinyangwe et al. (2004)
Tanzania	Tc	I, D	Mbwambo et al. (1988)
Uganda	Tb	D, I	Kazibwe et al. (2009)
Zimbabwe	Tc	D	Joshua et al. (1995)
Kenya	Tc	I	Gray et al. (1993)

Tc: *T. congolense*; Tb: *T. brucei*; Tv: *T. vivax*; I: isometamedium; D: dimenazene; ID: both isometamedium and dimenazee.

Delespaux et al., 2008).

As it is suggested by Delespaux et al. (2008), it is suspected that in several other African countries, resistance is present but is yet to be demonstrated, because in several countries surveys for resistance have not yet been carried out or cases of resistance have not been published (Melaku and Birrasa, 2013).

Eze et al. (2015) conducted a study on trypanocidal resistance in trypanosomes isolated from animals to diminazene and isometamidium, in sub humid tropical zone of Southeastern Nigeria. Their results of the sensitivity tests showed that out of the ten isolates tested, nine were resistant to diminazene aceturate at the dose of 7.0 mg/kg out of which four were slightly resistant whereas all the isolates were sensitive to diminazene at 28 mg/kg. On the other hand, only two isolates were resistant to isometamidium chloride at the dose of 1 mg/kg out of which one was slightly resistant.

Clausen et al. (1988) and Grace et al. (2009) reported for the presence of multiple drug resistant *T. congolense* in tsetse infested areas of Burkina Faso. Trypanocidal drug resistance appears to be widespread in the Adamaoua, Cameroon. Apparently, the problem is more serious in *T. congolense* than in *T. brucei*, which is unfortunate because the former is more pathogenic to cattle than the latter.

Qadeer et al. (2015) tested the sensitivity of trypanosome isolates from Nigeria in experimentally induced trypanosomiasis, and their result showed the presence of resistant *T. vivax* strains to a particular dosage of 3.5 mg/kg diminazene aceturate and to all dosages of isometamidium. Some reports on resistant trypanosomes are shown in Table 1.

The two important drugs (dimenazene and isometamidium) have been used for more than 40 years in Ethiopia (Dagnachew et al., 2015). Some studies conducted on the few isolates of *T congolense* in Ethiopia have shown the prevalence of drug resistance that might pose a higher risk for the tsetse infested areas of the countries (Abebe and Jobre, 1996). Moreover, Afework et al. (2000) and Tewelde et al. (2004) have conducted a large scale survey demonstrating area-wide resistance in at least one region of the country.

The magnitude of drug resistant trypanosomes across Ethiopia is not well documented (Chaka and Abebe, 2003). The dynamic nature of the epidemiology of drug resistant infection in the Ghibe valley, which was reported to be 6% in 1986 and i1989, is increased to 14% (Rowlands et al., 1993). In addition, Mulugeta et al. (1997) indicated a long-term occurrence of *T. congolense* resistant to diminazene, isometamidium and homidium in cattle of Ghibe, Ethiopia.

Afework et al. (2000) detected for the presence of resistance in mice using clones of *T. congolense* which were obtained from the isolates collected from relapsed chattels treated with ismetamidium, in northern western Ethiopia. Those clones found were resistant to both diminazene and isometamidium.

In Tigray, Ethiopia, *T. vivax* has developed resistance to dimenazene and iseomtamidum (Wlyohannes et al., 2010). According to the study, relapse occurred to diminazene aceturate and isomethamidum chloride at 3.5 and 0.25 mg/kg of body weight, respectively.

Different authors have reported the development of drug resistance trypanosomes in Gibhe valley (Codjia et al., 1993; Shimelis et al., 2008).

The four isolates of *T. congolense* from Ghibe, Bedelle, Sodo and Arbaminch regions of Ethiopia were found to be resistant to the curative action of diminazene (in mice and cattle) and isometamidium (in cattle) at a dose rate of

3.5 and 0.5 mg/kg body weight, respectively (Chaka and Abebe, 2003).

Recently, Hagos et al. (2014) tested sensitivity of *T. congolense* field isolates to isomethamidium and diminazene in Konso district, Southern Ethiopia. The demonstration of resistance to diminazene was about 33% from their study, which also could be to isometamidium. Current *T. congolense* isolates resistance was unsurprisingly an expected outcome.

Dagnachew et al. (2015) also conducted *in vivo* testing of two isolates of *T. vivax* from tsetse infested and non-infested areas of Ethiopia. The authors concluded that for the presence of drug resistance *T. vivax* (more than 20 percent) against recommended doses of both the available trypanocidal drugs.

CONCLUSION

Treatment of trypanosomiasis is currently facing a number of problems including toxicity of trypanocidal drugs and development of resistance by the parasites. These limitations have prompted the search for alternative active substances (such as of natural origin). Different factors can lead to the development of trypanocidal resistance including the sub-curative dose utilization and mutagenic ability of some of the drugs .Calf mortality can be high, with increased incidence of abortion and the financial and economic returns were also affected due to the prevalence drug resistant strains of trypanosomes. Safeguarding of the currently available drugs is mandatory to preserve the effectiveness of the drugs.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Metagenomic analysis of enteric bacterial pathogens affecting the performance of dairy cows in smallholder productions systems

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There is little information about the diversity of bacterial pathogens present in the rumen and feces of healthy cow and the subsequent effects on the performance of the host animal. The objectives of the present study were to genetically characterize the enteric bacterial pathogens found in the rumen fluid and cow feces and to identify the resistant genes responsible for antimicrobial resistance in the detected pathogens. The cow feces and rumen fluid samples (6 rumen fluid and 42 feces) were collected from lactating dairy cows. Using next generation sequencing, the enteric bacterial pathogens detected were screened for antimicrobial resistance genes using ResFinder-2.1 database in the center of Abricate. The characterized enteric bacterial pathogens include *Escherichia coli, Salmonella enterica, Streptococcus agalactiae, Streptococcus pyogenes, Campylobacter coli,* and *Campylobacter fetus* among others. Those enteric bacterial pathogens were also drug resistant bacteria except *Campylobacter coli*. The *Campylobacter fetus fetus* was identified as the only multidrug resistant bacterial pathogen detected in the cow feces. However, the abundant resistant genes detected confer resistance to tetracycline (17 genes from 209 contigs), beta-lactam (21 genes from 67 contigs), streptomycin (6 genes from 153 contigs), and sulfamethoxazole (2 genes from 72 contigs). This is the first study to identify the diversity of enteric bacterial pathogens from the station based and smallholder dairy cows in Kenya and Tanzania, respectively.

Key words: Antimicrobial resistant genes, enteric bacterial pathogens, dairy cows, next generation sequencing.

INTRODUCTION

Bovine ruminant particularly dairy cow contributes to the nutrition and wellbeing of humans' world widely by providing a variety of dairy products such as milk and its derivatives (Zhu, 2016). Milk production is of great economic concern to farmers and the quality is closely associated with the health of human beings.

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Improvement of its production in ruminant research relies on our understanding of the “microbial organ” of dairy cows (Petri et al., 2013; Zhu, 2016). Dairy cows have evolved a symbiotic relationship with a complex microbiome consisting of bacteria, fungi, and protozoa located in the reticulo-rumen that breakdown ingested food (Ross et al., 2012; Creevey et al., 2014; Pitta et al., 2016). These gut microbiome confer metabolic and immunological benefits to the host animal (Peng et al., 2015).

The rumen microbiota adapts rapidly to the intervention methods such as dietary formulations, biological feed additives and chemo-genomics of the host animal (Roehe et al., 2016). Therefore, nutrition represents an important tool for manipulating the microbial ecosystem to optimize rumen function while producing high-quality meat and milk for human consumption (Loor et al., 2016). However, the molecular characterization of the rumen microbiota is a viable option with regards to their effects on performance of dairy cows. Moreover, those animals produce large quantities of manure with a wide variety of pathogenic or non-pathogenic microorganisms to the dairy cows (Manyi-Loh et al., 2016). The bacterial pathogens found in the rumen nutrients are absorbed in the blood system and cause reproductive tracts infections such as mastitis and retained placenta (Wang et al., 2013) that affects the dairy industry (Wang et al., 2013). However, the techniques for identifying unidentified bacterial pathogens depend on the specific requirements for the species in the laboratories. The routine diagnostic techniques use different culturing methods, media, and reagents (Nakamura et al., 2008). Various bacteria require specific growth conditions and fail to grow in a given culture medium which leads to difficulties in handling samples in clinical microbiology laboratories (Castillo et al., 2006).

Furthermore, the feces shed from the gastro-intestinal tract (GIT) of the dairy cow are used in the agricultural farming. However, it presents the main source of antimicrobial resistance genes to the smallholder farms and the host animal (Zhang et al., 2015). The resistance genes are horizontally transferred in the bacterial species in the gut before fecal excretion (Chambers et al., 2015). Resistant genes excreted from animal gut contaminate the farms and may reach the human population through the consumption of dairy products. The use of the antimicrobials especially at sub therapeutic levels in the dairy farms selects for antimicrobial resistant (AMR) bacteria which contain the AMR genes (Sawant et al., 2007; Akindolire et al., 2015; Cameron and McAllister, 2016). The antimicrobial resistant pathogens contribute to increased mortality and morbidity of dairy cattle in the cattle production system which causes significant losses to dairy farmers (Call et al., 2008). The identification and characterization of the resistance genes present in the gut microbiome of the host animal was previously performed with routine diagnostic techniques such as the conventional culture methods, and were found to be insufficient and informative (Sawant et al., 2007).

Moreover, we depend on antimicrobials for the treatment of dairy cattle affected with pathogenic microorganisms (Idriss et al., 2014). The indiscriminate use of these antimicrobials in dairy cows benefits the development of resistant strains in the host animals (Idriss et al., 2014). However, little information is known about the source, diversity and distribution of antimicrobial resistance genes in the most non-culturable environmental bacterial pathogens (Chambers et al., 2015). Therefore, the use of bioinformatic approaches together with high-throughput sequencing techniques (HTS) in the analyses of microbiome overcome various methods used in the characterization of environmental microbes (Jami et al., 2014). Additionally, the use of next generation sequencing (NGS) techniques, bioinformatic tools, and molecular based approaches is the potential laboratory way for studying the diversity of rumen microbiota of health individuals (Dowd et al., 2008). The HTS techniques (Illumina sequencing and Roche/454 pyrosequencing) are mainly used in the detection and characterization of diversity of microbiomes by analyzing 16S rRNA gene amplicons in the current decade (Klein-Jöbstl et al., 2014). However, the analyses of data obtained from these sequencing technologies need advanced computational approaches and powerful machines (Jovel et al., 2016). These cause problems for microbiologists and laboratory clinicians when studying the diversity of microbiome (Jovel et al., 2016).

In this regards, metagenomics, the genomic analysis of population of microorganisms makes possible the profiling of environmental microbiome (Bashir et al., 2014; Flygare et al., 2016). Metagenomics allows the identification and characterization of the composition of microbiota as well as the abundance of their genes (Roehe et al., 2016). However, this platform provide an important advantage as the single DNA fragments of a library is sequenced directly without cloning and compares the sequences to known sequence database (Barzon et al., 2011; Nathani et al., 2013). Therefore, the objectives of the present study were to identify and genetically characterize the enteric bacterial pathogens found in the cow feces and rumen fluid of dairy cows affecting their performance using metagenomic approaches. In addition, it also identified the genes responsible for antimicrobial resistance in the detected bacterial pathogen.

MATERIALS AND METHODS

Ethics statement

The study was approved by the institutional ethics committee of the University of Nairobi (UoN), Faculty of Veterinary Medicine guidelines and the International Livestock Research Institute (ILRI)-Institutional Animal Care and Use Committee (IACUC). The study was conducted in accordance to the good scientific practices

approved by the two institutions. The animals were restrained by the experienced veterinary professionals during the data collection to reduce the discomfort.

Study sites

This study was carried out in one site in Kenya (University of Nairobi (UoN) Faculty of Veterinary Medicine farm) and two study sites in Tanzania namely; Lushoto and Rungwe. The UoN Faculty of Veterinary Medicine farm is located on a 375 acre piece of land in Kanyariri Village of Kiambu County in Kenya at latitude 1°14'33.4"S and longitude 36°42'36.3"E (https://www.uonbi.cavs.ac.ke). Lushoto district is located in Tanga region which lies at latitudes 4° and 6°S and longitudes 38° to 39°E (Mfune, 2015). Rungwe district lies between latitudes 9° 00 and 9° 30 E and longitudes 33 °E and 34°S in Mbeya region (Karwani et al., 2016).

Animals and experimental treatments

Six adults lactating dairy cows reared at the UoN Faculty of Veterinary Medicine farm were used in the experiment. The criteria for selection were based on the breed, body condition, medical history and cows in their first stage of lactation. Two lactating dairy cows were selected in each genotype namely Jersey, Friesian and Jersey×Friesian cross. The animals had an average body weight ± standard deviation of 300 ± 50 kg and were 174 ± 15 days into their first lactation. The experimental animals were assigned to a completely randomized design (CRD) experiment with a 3 × 3 factorial arrangement of treatments. The treatments were three cattle genotypes (Friesian (Fri), Jersey (Jer) and Friesian X Jersey cross (CB)) and three diets (90% crop residue and 10% concentrate, followed by 75% crop residue and 25% concentrate and then 60% crop residue and 40% concentrate). The three rations were formulated to meet the energy requirements of cows yielding 20 kg of milk/day with 4.0% milk fat and 3.5% true protein, by the NRC - Nutrient Requirements of Dairy Cattle Software v 1.9 (NRC, 2001). Feeds were offered *ad libitum* as a total mixed ration (TMR) to avoid the selection of dietary components. The dietary components for the crop residue were: Rhodes grass (*Chloris gayana*) ray, rapier grass (*Pennisetum purpureum*), kikuyu grass (*Pennisetum clandestinum*), maize (*Zea mays*) stover. These were mixed with dairy meal and urea at different proportions to make the three diets. The chemical composition of the dietary components was assayed according to the Association of Official Analytic Chemist (AOAC) methods (1998) while the dietary fiber determination was conducted according to Van Soest et al. (1991).

After a 10-day acclimatization period, the cattle were fed the three different diets in three consecutive 10-day periods. Experimental diets were offered in two meals at 8am and 6pm, one-half of the allowed daily rations at each feeding. Throughout the 30 days of the experiment, the cattle were housed in stalls and given free access to fresh water and mineral supplement.

Sample collection

Two sample types (fecal grab and rumen fluid) were collected from each experimental animal. Rumen samples were collected via a flexible stomach tube while fecal grabs were collected from the rectum. Furthermore, thirty six (36) fecal samples were collected from selected animals in Lushoto and Rungwe districts in Tanzania. Approximately, 250 to 500 g of individual fecal samples were collected from the rectum of each cattle using a clean palpation sleeve and sterile lubricant for each collection and a sub-sample transferred into sterile 50 ml falcon tube (Chambers et al., 2015). In total, four serial samples (for each sample type) were collected from each of the experimental animals during the feeding experiment. The samples were collected at days 0, 10, 20 and 30 (that is, on the last day of the 10 days on each experimental feed). Samples were collected approximately 2 h after the morning feeding. The samples were immediately kept on ice in the cool box and shipped to the Biosciences east and central Africa (BecA-ILRI) Hub, laboratory at the International Livestock Research Institute where they were stored at -20ºC until microbial DNA analysis. Frozen samples were thawed at room temperature before being mixed thoroughly by vortexing for 30 s at maximum speed.

DNA extraction and library construction with Illumina sequencing

Total genomic DNA was extracted from all samples using the commercially available QIAamp DNA Stool Mini Kit (Qiagen, USA) according to the manufacturer's instructions but with a few modifications. The modifications included: (i) Using double the recommended sample volume and (ii) addition of 2 µl of RNAse A after mixing propeinase K and the sample. The DNA concentration and quality were assessed by Nanodrop spectrophotometry (Nanodrop Technologies), Qubit® 2.0 Fluorometer with the Qubit ®dsDNA HS Assay Kit and agarose gel electrophoresis (Onate et al., 2015). The recovered DNA products were stored at -20°C until further analysis. Next, the Nextera XT DNA library preparations was performed following the workflow and protocol described by Kim et al. (2013) followed by Illumina Miseq Sequencing. Briefly, 50 ng of genomic DNA were first tagmented in a transposase-mediated reaction that simultaneously fragments and tags DNA with adapters. The adapter-tagged DNA fragment libraries were purified with Zymo Kit to remove unwanted constituents from the tagmentation reaction. Subsequently, the sequencing adapters were added to the fragment library by limited-cycle PCR, and finally the DNA was size-selected for sequencing and finally paired end sequencing was performed using the Illumina MiSeq v3 (Illumina) System.

Quality control of the raw sequence reads and K-mer analysis

In this study, 48 samples (12 were collected at the UoN Faculty of Veterinary Medicine farm and 36 from the smallholder farms in Tanzania) were used. The quality of the data was checked using fastQC/v0.11.2. Then, Sickle/v1.33 was used for trimming of the low quality reads at the length threshold of 100 bps and the quality threshold of 20 (Q=20). Thereafter, K-mer analysis of these raw NGS sequence reads was determined prior to filtering and functional annotation of the reads (Onate et al., 2015). Kmergenie/v1.7044 (Sievers et al., 2017), an efficient single program written in C/C++, was used for this process. The frequencies of different k-mer abundance value contained in a set of reads were plotted as a k-mer abundance histogram (Chikhi and Medvedev, 2013; Onate et al., 2015). Finally, the optimal k value that maximizes the number of genomic k-mers (Chikhi and Medvedev, 2013) was k=23 (optimal k-mer) was identified.

Metagenomic assemblies of the reads and taxonomic annotation of the contigs

The *De novo* assembly of the quality filtered reads after trimming, was performed using the Ray/v2.3.1 to give the larger fragments technically known as contigs and scaffolds in fasta files. The functional annotation of the contigs was performed using Prokka homology-search against the protein reference in the Diamond database. However, the taxonomic annotation and analysis was

done using the CAT (Contig Annotation Tool) pipeline utilizing the rapid prokaryotic genome annotation (Prokka) described in details by Seemann (2014).

Taxonomic characterization and detection of enteric bacterial pathogens

The taxonomic visualization of the bacterial species present in the metagenome was performed using Krona tool/v 2.7. The classified contigs representing the cow rumen fluid and feces at the UoN Faculty of Veterinary Medicine farm and cow feces at the smallholder farms (Lushoto and Rungwe), respectively were assembled together and one Krona graph was made for each group. The enteric bacterial pathogens were identified through a literature search using dendrograms at the scholarly Google database.

Identification of antimicrobial resistance genes in the enteric bacterial pathogens

The characterization and annotation of resistant genes responsible for antimicrobial resistance in the bacterial pathogens were carried out using the Abricate database. The Abricate annotation pool used the ResFinder-2.1 dataset to identify and annotate potential antimicrobial resistance genes using BLAST similarity search (https://github.com/tseemann/abricate). The Abricate using the ResFinder selects the percentage identity (ID) thresholds that are identical between the best matching resistance genes and the corresponding sequence in the genome (Zankari et al., 2012). The default ID is 100%. These provided the type of antimicrobials in which are present and the accession numbers in the GenBank. Additionally, the pathogenic bacterium with multidrug resistance was identified by accessing the GenBank number provided by the ResFinder-2.1 in the National Center of Biotechnology Information (NCBI).

RESULTS

Characterization of enteric bacterial pathogens of economic importance identified in the rumen fluid and feces of cows raised at the station and smallholder farms

The study identified a high prevalence of enteric bacterial pathogens. These pathogens were characterized using dendrogram representations of rumen fluid and fecal samples. The bacterial species in each bacterial family present in the dendrograms were reviewed using scholarly Google database to identify those existing in rumen fluid and feces. Within each family, the highest number of bacterial species was detected in *Enterobacteriaceae* (five species) followed by *Streptococcaceae* and *Campylobacteriaceae* (three species each), *Staphylococcaceae* and *Enterococcaceae* (two bacterial species each) (Table 1). Both *Streptococcaceae* and *Enterococcaceae* families had two bacterial species detected in the rumen fluid and all the fecal samples. The *Enterobacteriaceae*, *Bacteroidaceae*, *Bacillaceae*, and *Prevotellaceae* had one species each detected in all the samples. The *Staphylococcaceae*, *Clostridiaceae*, and *Listeriaceae* were isolated from all the samples at the smallholder farms, whereas the *Mycoplasmataceae* and *Campylobacteriaceae* (*Campylobacter coli*) were only identified at the UoN Faculty of Veterinary Medicine farm samples. The bacterial species present in the fecal samples at the station farm were also detected in the rumen fluid, except *Campylobacter fetus, Shigella flexneri, Mycoplasma pneumoniae* and *Vibrio cholera.* More bacterial species were detected in fecal samples from smallholder dairy cows in Rungwe than in Lushoto. Specifically, *Streptococcus pneumoniae*, *Shigella dysenteriae* and *Clostridium perfringens* were only detected in fecal samples from Rungwe farm (Figure 1).

The enteric bacterial pathogens of economic importance were also determined by checking the number of counts (hits in the contigs) of bacterial pathogens through opening the dendrogram representations at the bacterial species level. The bacterial pathogens from bacterial families of *Enterobacteriaceae*, *Campylobacteriaceae*, *Bacteroidaceae*, and *Prevotellaceae* indicated the highest number of contigs in their genomes in all the samples. Bacterial pathogens from the feces recorded a high number of contigs than the rumen fluid samples. This is in agreement with the number of bacterial pathogens detected also both in feces and rumen fluid. Pathogenic species *Escherichia coli* and *Prevotella ruminicola* displayed the highest number of counts (185 and 220 respectively) in feces at Rungwe smallholder farms and rumen fluid samples respectively. Within the feces, samples from Tanzania had a relatively higher abundance in bacterial pathogens compared to those from the UoN station farm. At the Tanzania smallholder farm level, the bacterial pathogens detected in fecal samples from Rungwe indicated a relatively higher abundance than those sampled from Lushoto (Table 1).

Antimicrobial resistance (AMR) genes identified in the enteric bacterial pathogens

All the contigs (assembled genomes) were blasted against the ResFinder-2.1 database at the center of Abricate and the AMR genes were identified based on the similarity of genes present in the GenBank database. The results obtained show that there were 97 resistance genes in cow fecal samples and 8 resistance genes in the rumen fluid at UoN Faculty of Veterinary Medicine farm. The analysis of the fecal samples from the smallholder farms in Tanzania revealed a total of 295 and 307 resistance genes in Lushoto and Rungwe sites, respectively. The most abundant resistance genes detected in the enteric bacterial pathogens in this study confer resistance to beta-lactam (21 genes) and tetracycline (17 genes) drugs. The rest are shown in Table 2. The tetracycline and beta-lactam resistance genes were detected in all the fecal samples.

Table 1. Number of contigs with enteric bacterial pathogens of economic importance identified in the rumen fluid and feces of cows kept at the station (University of Nairobi) and smallholder (Lushoto and Rungwe) farms.

Bacterial family	Bacterial species	Rumen fluid*	Feces		
		UoN**	UoN**	Lushoto**	Rungwe**
Enterobacteriaceae	*Escherichia coli*	2	3	40	185
	Salmonella enterica	-	-	5	9
	Klebsiella pneumonia	-	-	2	19
	Shigella flexneri	-	1	1	16
	Shigella dysenteriae	-	-	-	5
Streptococcaceae	*Streptococcus agalactiae*	1	3	1	1
	Streptococcus pyogenes	1	2	2	1
	Streptococcus pneumonia	-	-	-	1
Campylobacteriaceae	*Campylobacter fetus*	-	6	41	27
	Campylobacter coli	1	8	-	-
	Campylobacter jejuni	-	-	37	31
Staphylococcaceae	*Staphylococcus aureus*	-	-	3	3
	Staphylococcus sciuri	-	-	1	2
Enterococcaceae	*Enterococcus faecium*	1	3	12	2
	Enterococcus faecalis	1	5	7	5
Clostridiaceae	*Clostridium botulinum*	-	-	21	4
	Clostridium perfringens	-	-	-	3
Bacteroidaceae	*Bacteroides fragilis*	39	45	16	50
	Bacteroides pyogenes	-	-	6	-
Bacillaceae	*Bacillus cereus*	2	6	1	5
Prevotellaceae	*Prevotella ruminicola*	220	18	5	10
Mycoplasmataceae	*Mycoplasma pneumonia*	-	1	-	-
Listeriaceae	*Listeria monocytogenes*	-	-	2	1
Vibrionaceae	*Vibrio cholera*	-	1	1	1

*Specimens of rumen fluid only collected from the University of Nairobi (UoN) station farm; **Number of contigs of bacterial specie in the sample;
**-: absence of bacterial specie in the sample.

Streptomycin, Sulfamethoxazole, Quinolone and Chloramphenicol resistance genes were only detected and highly prevalent in the smallholder farms.

Characterization of the drug resistant bacterial pathogens identified in the GenBank

The drug resistant bacterial pathogens were identified and characterized according to the similarity search in the GenBank. The antimicrobial resistant bacterial pathogens isolated from the cow feces at the UoN Faculty of Veterinary Medicine farm and smallholder farms in Tanzania are reported in Table 3. The most prevalent drug resistant bacterial pathogens were detected from *Enterobacteriaceae*, *Streptococcaceae*, *Campylobacteriaceae*, *Staphylococcaceae* and *Enterococcaceae*.

DISCUSSION

The highest number of bacterial species was detected in the *Enterobacteriaceae* followed by *Streptococcaceae*, *Campylobacteriaceae*, *Staphylococcaceae* and *Enterococcaceae* and the rest of the families had a species each (Table 1). The predominant *Enterobacteriaceae* and *Streptococcaceae* bacterial families are Gram negative and Gram positive respectively and are associated with brucellosis, pneumonia, salmonellosis, clinical and subclinical mastitis diseases predominantly reported in smallholder farms in the tropics. There are economically important diseases of livestock causing reproductive wastage through infertility, delayed heat, loss of calves, reduced meat and milk production, culling and economic losses from international trade bans of infected dairy products (Hossain et al., 2017). The *Staphylococcaceae*,

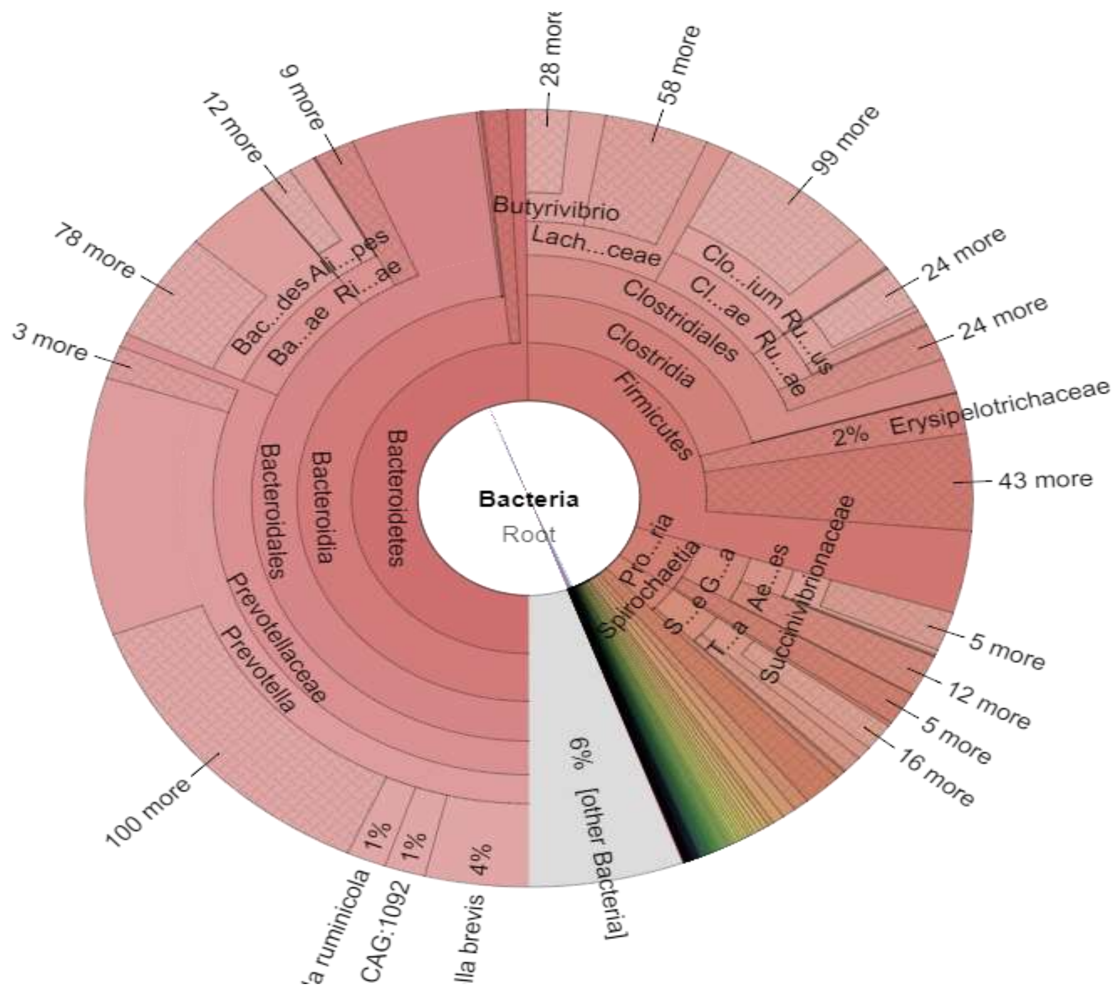

Figure 1. The taxonomic characterization of the rumen fluid bacterial species identified at the UoN Faculty of Veterinary Medicine farm.

Clostridiaceae and *Listeriaceae* bacterial families were detected in samples from smallholder farms, whereas the *Mycoplasmataceae* (*Mycoplasma pneumoniae*) and *Campylobacteriaceae* (*Campylobacter coli*) were identified at the station samples. There were differences observed between rumen fluid and feces, and also within fecal samples. The major bacterial pathogens identified from smallholder farms in this study, which included *Salmonella enterica*, *Klebsiella pneumoniae, Streptococcus sciuri*, *Campylobacter jejuni,* and *Staphylococcus aureus*, among others were in agreement with the findings reported by Osman et al. (2009) and Sharif and Muhammad (2009).

Additionally, these pathogens have been reported to cause clinical and subclinical mastitis (Idriss et al., 2014; Thompson-crispi et al., 2014; Abebe et al., 2016). The high presence of the enteric bacterial pathogens in the feces than the rumen fluid collected from the same cow indicates that most bacterial pathogens colonize the lower sections of gastro-intestinal tract of the animal such as colon, cecum, ileum and jejunum (Gerzova et al., 2015).

In the *Enterobacteriaceae*, *Escherichia coli*, *Salmonella enterica* and *Klebsiella pneumonia* was detected. *E. coli* is among the zoonotic bacterial pathogens that cause subclinical mastitis and commonly affects dairy cows during parturition leading them to the local or acute mastitis (Sandra Bjork, 2013; Osman et al., 2014; Madoshi et al., 2016; Hintong et al., 2017). It is excreted in the feces of healthy animal and spreads to the farm via soil or water (Amadi et al., 2015). This pathogenic bacterium was also identified in the cow fecal samples in other studies by Pandey at al. (2015), Madoshi et al. (2016) and Bako et al. (2017). Furthermore, *E. coli* were identified also by Megersha et al. (2009) and Amadi et al. (2015) in Ethiopia and Grenada in the feces of sheep and goats, respectively. In the present study, *K. pneumoniae* was detected as well. This bacterial pathogen was detected also in fecal samples of dairy cattle in other studies by Munoz et al. (2007), Sandra Bjork (2013),

Table 2. Number of contigs with antimicrobial resistance genes identified in the feces of cows kept at the station (University of Nairobi) and smallholder (Lushoto and Rungwe) farms.

Antimicrobial drug	Gene	Number of contigs (counts)		
		UoN	Lushoto	Rungwe
Tetracycline	*tet(32), tet(34), tet(35), tet(36), tet(37), tet(40), tet(44), tetA(P), tet(A), tet(C), tet(G), tet(H), tet(O), tet(Q), tet(S), tet(X), tet(W)*	56	64	89
Beta-Lactam	*blaACC-1, blaACC-2, blaBES-1, blaCMY-19, blaCMY-110, blaFAR-1, blaGOB-17, blaOXA-2, blaOXA-50, blaOXA-141, blaOXA-164, blaOXA-209, blaOXA-347, blaPAO, blaRHN-1, blaTEM-102, blaTEM-111, cfxA, cfxA2, cfxA3, cfxA6*	24	18	25
Streptomycin	*StrA, StrB , ant(6)-Ib, aadA17, aadE, aadK*	-	80	73
Sulfamethoxazole	*Sul1, Sul2*	-	37	35
Quinolone	*QnrB4, qepA, oqxB*	-	35	33
Chloramphenicol	*CatA, CatB, cmlA, floR*	-	27	25
Streptomycin and spectinomycin	*aadA1, aadA2, aadA4, aadA6*	-	11	9
Vancomycin	*VanG, VanR-D, VanR-F, VanR-G, VanS-G, VanY-Pt, vat(B), vat(E)*	1	5	3
Trimethoprim	*dfrB1, dfrG*	-	4	4
Lincomycin	*Lnu(C)*	5	-	1
Gentamicin	*aac(6')-aph(2")*	2	1	2
Neomycin	*aph(3')-Ia, aph(3')-Ic*	-	3	2
Erythromycin	*Ere(A), erm(F)*	-	2	2
Oxazolidinone and phenicols	*optrA*	-	1	1

*Rumen fluid: Eight (8) resistance genes detected from bacterial pathogens in the rumen fluid at the University of Nairobi (UoN) were not reported because of their low abundance; -: absence of resistance genes.

Mansour et al. (2014) and Osman et al. (2014).

In the *Streptococcaceae*, *Streptococcus agalactiae* and *Streptococcus pyogenes* were detected in all the samples while *S. pneumoniae* was only present at Rungwe on-farms (Table 1). The Streptococcal species identified in the present study were lower than those reported by Mekibib et al. (2010) when studying bacterial pathogens causing mastitis in dairy cattle farms in Central Ethiopia. In the *Campylobacteriaceae*, *C. coli, C. fetus*, and *C. jejuni* were detected in cow rumen fluid and feces (Table 1). Similar findings were reported in Tanzania, Kenya and Ghana by (Kashoma et al., 2015; Nguyen et al., 2016; Karikari et al., 2017) in beef cattle feces, faeces and cloacal swabs of chickens and faeces and carcasses of healthy livestock animals, respectively. Pathogenic bacteria *Staphylococcus aureus* and *Staphylococcus sciuri* were detected only at the smallholder farms in Tanzania (Table 1). These bacterial pathogens which cause clinical and subclinical mastitis were also detected in the fecal samples in other studies by Mekibib et al. (2010), Sandra Bjork (2013) and Abo-Shama (2014). Finally, *Enterococcus* and *Clostridial species* were also detected. The pathogenic *E. faecalis* and *E . faecium* were detected both in the rumen fluid and feces. Similar findings were also reported by Goskel et al. (2016) and Beukers et al. (2017). They were also reported infecal and cecal samples of chickens by Diarra et al. (2010). Furthermore, *Clostridium botulinum* and *Clostridium perfringens* were detected in fecal samples from the smallholder farms. These findings are in agreement with the results reported by Ahsani et al. (2010), Kruger et al. (2011), Neuhaus et al. (2015) and Fohler et al. (2016) in animal feces and liquid manure from dairy cows.

In the current study, the antimicrobial resistance genes identified and characterized from the station and on-farms were presented in Table 2. The abundant resistant genes identified, confer resistance to tetracycline, beta-lactam, streptomycin, sulfamethoxazole, Quinolone and chloramphenicol drugs. Similar findings were reported by Thames et al. (2012) who reported the *tetC, tetG, tetO, tetW,* and *tetX* as antimicrobial resistance genes corresponding to tetracycline, *ermB, ermF* for macrolide, and *sul1* and *sul2* for sulfonamide identified from dairy calves manures. These findings are in agreement with the results by Agga et al. (2015), Gerzova et al. (2015), Iweriebor et al. (2015), Madoshi et al. (2016) and Pitta et

Table 3. Drug resistant bacterial pathogens of economic importance (including zoonotic) identified in feces of cows kept at station (University of Nairobi) and smallholder (Lushoto and Rungwe) farms.

Bacterial family	Bacterial species	Feces			Accession number
		UoN	Lushoto	Rungwe	
Enterobacteriaceae	*Escherichia coli*	-	+	+	AY224815
	Salmonella enterica	-	+	+	AY963803
	Klebsiella pneumonia	-	+	+	AB194410
	Shigella flexneri	-	+	+	AF321551
Streptococcaceae	*Streptococcus agalactiae*	+	+	+	AY928180
	Streptococcus pyogenes	+	+	+	AF227521
Campylobacteriaceae	*Campylobacter fetus*	+	+	+	FN594949
	Campylobacter jejuni	-	+	+	KF652095
Staphylococcaceae	*Staphylococcus aureus*	-	+	+	AJ579365
	Staphylococcus scriuri	-	-	+	U194559
Enterococcaceae	*Enterococcus faecalis*	+	+	+	AY271782
	Enterococcus faecium	+	-	+	KF421157
Clostridiaceae	*Clostridium perfringens*	-	-	+	L20800
Prevotellaceae	*Prevotella ruminicola*	+	+	+	L33696

+: Presence of drug resistant bacterial pathogens in the sample; -: Absence of drug resistant bacterial pathogens in the sample.

al. (2016) but are in contrast with the findings by Ahmed and Shimamoto (2011) and Chandra et al. (2014) who reported *blaCTX-M, blaTEM*, *blaCMY, blaSHV and blaOXA* as the predominant genes in their studies. The presence of a high number of genes that confer resistance to these antimicrobials can be explained by indiscriminate use of these drugs in the treatment of diseased animals, prevention of diseases in the farms or with their use as growth promoters in animal feed production (Sawant et al., 2007; Akindolire et al., 2015; Pandey et al., 2015; Cameron and McAllister, 2016). Furthermore, these drugs are cheap, widely available on the markets and have few predilection sites of administrations (Sawant et al., 2007; Osman et al., 2014; Beyene et al., 2017). The difference in the abundance of AMR genes was observed between the rumen fluid and cow feces isolates at the station farm (Table 2). The difference in AMR gene abundances also was observed between the isolates identified at the station and on-farms (Table 2). This difference could be due to differences in geographical locations, environment, management, farming practices, and concentration of the farms in the locations as reported by Kashoma et al. (2015) and Nyabundi et al. (2017). Additionally, the samples originating from different environment display different AMR gene abundance as reported by Gerzova et al. (2015). The present study identified the abundance of tetracycline resistance genes from cow fecal isolates. These findings are in agreement with the results by Thames et al., (2012), Agga et al. (2015), Gerzova et al. (2015), Iweriebor et al. (2015), Madoshi et al. (2016) and Pitta et al. (2016) who reported high presence of *tetC, tetG, tetO, tetW,* and *tetX* in their studies. The findings are also similar to the results reported by Kyselkova et al. (2015) when studying the occurrence of tetracycline resistance genes at conventional dairy farm. However, Englen et al. (2006) reported that *Campylobacter jejuni* displayed tetracycline and nalidixic acid resistance genes while *C. coli* indicated resistance to azithromycin, ciprofloxacin, clindamycin, erythromycin, gentamicin, and tetracycline from cow fecal isolates.

Moreover, beta-lactam resistance genes were also identified in the present study. The commonly identified AMR genes from cow fecal microbiota at the UoN Faculty of Veterinary Medicine farm were beta-lactam resistance genes (*cfxA_1, cfxA2_1, cfxA3_1 and cfxA6_1).* The high presence of *cfxA2*, and *cfxA3* resistance genes are in agreement with the findings by Chambers et al. (2015). The smallholder farms were dominated by bacteria showing beta-lactam resistance genes other than that detected at the station farm (Table 2). These findings are in agreement with the results reported by Ahmed and Shimamoto (2011), Jiang and Zhang (2013), Chandra et al. (2014) and Olowe et al. (2015). Furthermore, these findings are in contrast with the results reported by Mir et al. (2016) who reported high abundance of *blaTEM* and *blaCTX-M* genes detected from cefotaxime resistant bacteria in the cow feces. In this study, there was a difference between resistance genes identified at Lushoto and Rungwe smallholder farms. But no difference was

observed between Rungwe and UoN Faculty of Veterinary Medicine farms (Table 2). In the study on the prevalence of *E. coli* from dairy cattle feces in Eastern Cape, Iweriebor et al. (2015) reported that *E. coli* displayed resistance genes (*blaampc, blaCMY, blaCTX-M, blaTEM, tetA,* and *strA*) conferring resistance to beta-lactam, tetracycline, and streptomycin drugs, respectively. Similar findings were also reported in the current study. In the studies by Wittun et al. (2010), Schmid et al. (2013) and Gao et al. (2015), *E. coli* exhibited high presence of *CTX-M-14, CTX-M-15* and *TEM-52* genes from feces of dairy cattle and pig farms, respectively.

The findings from the present study revealed that streptomycin resistance genes (*StrA, StrB, ant(6)-Ib, aadA17, aadE, aadK)* were only present in the smallholder farms. These findings are in agreement with those reported by Srinivasan et al. (2007) in soils contaminated with bacterial pathogens in the dairy farm. The presence of *StrA* and *StrB* resistance genes in the current study are in agreement with the results reported by Aslam et al. (2010) from the cow feces infected with *E. coli* in Alberta, Canada. However, two sulfamethoxazole resistance genes which are *sul1* and *sul2* genes were also detected at the smallholder farms (Table 2). These findings are in agreement with the results by Gerzova et al. (2015) who reported the abundance of *strA, sul1*, and *sul2* genes in porcine fecal microbiota. In the present study, *QnrB4, qepA, oqxB* quinolone resistance genes were detected at the smallholder farms in Tanzania (Table 2). These findings are in contrast with the results reported by Zhang et al. (2015) who reported *qnrA* and *qnrS* genes detected in the pig fecal microbiota. However, no quinolone resistance gene was detected from cow fecal isolates at the UoN Faculty of Veterinary Medicine farm. No difference in abundance of quinolone resistance genes was observed between the isolates detected from cow feces at smallholder farms in Tanzania. In the study by Bae et al. (2005), the *C. jejuni* exhibited resistance to doxycline and *C. coli* showed resistance to quinolone antimicrobials detected also in the current study.

In conclusion, *CatA, CatB, cmlA, floR* chloramphenicol resistance genes from cow fecal isolates were identified in the smallholder farms (Table 2). Similar findings were reported by Sudda et al. (2016) in Tanzania. However, there was no chloramphenicol resistance gene identified at the station farm. The high abundance of chloramphenicol resistance genes at smallholder farms could be due to the smallholder farmers using the non-prescription drugs and not keeping treatment records about animals. Additionally, this presence could be attributed to its widespread and indiscriminate use in the treatment and prevention of diseases, or transfer of the resistant genes between animals, humans, and environments through the cross contamination (Omojowo and Omojasola, 2013; Sudda et al., 2016; Beyene et al., 2017; Messele et al., 2017).

Conclusion

This study results deepens our understanding of the diversity of enteric bacterial pathogens detected from cow rumen fluid and feces at the UoN Faculty of Veterinary Medicine farm and smallholder farms. It provided also insight on the prevalence of AMR genes from those enteric bacterial pathogens in the dairy cows whose cow feces is used to fertilize the farms. The characterized enteric bacterial pathogens of economic importance include *E. coli, S. enterica, K. pneumoniae, S. agalactiae, S. pyogenes, C. coli, C. fetus, C. jejuni, S. aureus, S. sciuri, E. faecalis, E. faecium, C. botulinum* and *C. perfringens*. Furthermore, the resistant genes detected in the enteric bacterial pathogens in this study confer resistance to tetracycline (17 genes from 209 contigs), beta-lactam (21 genes from 67 contigs), streptomycin (6 genes from 153 contigs), sulfamethoxazole (2 genes from 72 contigs), Quinolone (3 genes from 68 contigs) and chloramphenicol (4 genes from 52 contigs). Therefore, the identification of genes responsible for antimicrobial resistance in the bacterial pathogens may allow the development of novel clinical interventions against the GIT diseases of the dairy cows. The future studies are needed to identify the drug resistant bacterial pathogens with the spread of antimicrobial resistance in the farms. This will become a clear tool for developing the strategy to prevent the indiscriminate use of already resistant drugs in the farms.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Effectiveness of exopolysaccharides and biofilm forming plant growth promoting rhizobacteria on salinity tolerance of faba bean (*Vicia faba* L.)

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This study aimed to investigate the production of biofilm and exopolysaccharides by plant growth promoting rhizobacteria (PGPR) under different salt concentrations. In this study, the activity of biofilm formation and exopolysaccharides production by 20 strains of PGPR which previously isolated and identified from root samples of different crops were determined under different salt concentrations. Out of 20 strains, only 12 PGPR strains have the ability to form biofilm at 0.0, 50, 100 and 150 mM NaCl concentration. PGPR strains with the highest activity of biofilm formation and exopolysaccharides production were selected to check them on the faba bean plants under different concentration of salt stress. Inoculation with PGPR strains increased plant growth at higher level of salt concentrations compared with their corresponding uninoculated ones. The strain *Pseudomonas anguilliseptica* SAW 24 showed the highest activity of biofilm formation and exopolysaccharides production at different NaCl concentrations furthermore, gave the highest records of plant height (cm), fresh and dry weight (g/plant) of faba bean plants. The activities of biofilm formation and exopolysaccharides production of plant growth promoting bacteria enhance faba bean plants against different salt concentrations.

Key words: Biofilm, faba bean, exopolysaccharides, salinity, plant growth promoting rhizobacteria (PGPR).

INTRODUCTION

Biotic and abiotic stress factors have a major effect on plants which cause major damages to crop production around the world (Nemati et al., 2011). Salt stress considers one of the major problems that cause a decrease in fertile land productivity. Salinity not only affects in agriculture but also has other problems that effect on biodiversity of that environment. Unfortunately, leguminous crop is most salt-sensitive which severely affected by soil salinity throughout the world, to enhance its yield and productivity using safe biological measures to improve the soil management and symbiotic relationships (Sarker and Erskine, 2006; Fernandez-Aunión et al., 2010). Beneficial bacteria that enhance growth promotion and prime stress tolerance of plants

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have great chance to improve crop production and environmental friendly resource management. Aggregation of biofilm formation is commonly detected in bacteria. Biofilm is the aggregation of microbial cells that are irreversibly connected with biotic and abiotic surface and usually enclosed in the self-secreted extracellular polymeric substances.

Microorganisms within biofilms have a lot of advantages (Wilson, 2005; De Beer and Stoodley, 2006; Vu et al., 2009; Afrasayab et al., 2010; Nawaz and Ashraf, 2010; Asari, 2015) such as: protect the plant from external stress, Increase adhesion to surfaces, high population densities, high tolerance to antimicrobial agents and higher level of nutritional competition between microorganisms.

The main objective of this research was to check the biofilm formation and exopolysaccharides production by PGPR strains at different salt concentration and study the most efficient PGPR strains in terms of their ability to produce biofilm and exopolysaccharides under salt stress conditions on faba bean plants.

MATERIALS AND METHODS

Microorganisms and growth condition

Selection of the best biofilm forming bacteria was under different salt concentrations (0.0, 50, 100 or 150 mM). 20 strains of plant growth promoting rhizobacteria (PGPR) used in this study are presented in Table 1. These strains were previously isolated and identified by Fathalla et al. (2015).

The strains were screened for the potential of biofilm formation in nutrient broth (NB) which was prepared according to Difco (1985). The qualitative and quantitative biofilm formation assays were carried out as follows;

Qualitative assay for biofilm detection

A loopfull of bacterial strains were inoculated in 10 ml of NB with different NaCl concentrations (0, 50, 100 and 150 mM) in test tubes. The test tubes were incubated at 28°C for two days. Then, the supernatant was thrown away and the test tubes were washed with phosphate buffer saline (pH 7). The dried glass tubes were stained with 0.1% of crystal violet for 15 min and stain was removed by washing the tubes with distilled water. Biofilm formation in tubes was detected when a visible film lined the wall and the bottom of the tube.

Quantitative assay

The biofilm production was quantitatively assayed according to the method described by Arciola et al. (2002). Single colonies of strains from nutrient agar plates were inoculated in 10 mL of NB in separate test tubes, with different NaCl concentrations (0, 50, 100, 150 mM) through broth incubated for 2 days at 28°C and diluted (1 in 100) with fresh medium. Individual wells of sterile 96 well flat bottom polystyrene tissue culture treated plates were filled with 0.2 ml aliquots of the diluted cultures. Negative control wells contained sterile broth only.

Table 1. Plant growth promoting rhizobacteria strains.

Code	Strain
SAW19	*Pseudomonas putida* SAW19
SAW1	*Pseudomonas mosselii* SAW1
SAW7	*Pseudomonas entomophila* SAW7
SAC1	*Pseudomonas corrugata* AC1
SAW10	*Pseudomonas plecoglossicida* SAW10
SAB12	*Pseudomonas putida* SAB12
SAW23	*Pseudomonas corrugata* SAW23
SAW24	*Pseudomonas anguilliseptica* SAW24
SAW5	*Pseudomonas entomophila* SAW5
SAW9	*Pseudomonas argentinensis* SAW9
SAW22	*Pseudomonas putida* SAW22
SAB15	*Pseudomonas palleroniana* SAB15
SAW15	*Pseudomonas plecoglossicida* SAB1
SAB1	*Pseudomonas palleroniana* SAB17
SAB17	*Pseudomonas parafulva* SAB14
SAB14	*Pseudomonas putida* SAB12
SAB12	*Pseudomonas plecoglossicida* SAB8
SAB8	*Pseudomonas putida* SAW3
SAW3	*Pseudomonas putida* SAW3
SAB10	*Pseudomonas putida* SAB10

The plates were incubated at 28°C for two days. After incubation period, content of each well was gently decanted, then washed with phosphate buffer saline (pH 7) and stained by crystal violet (0.1%). Stain was removed by washing the wells with distilled water and dried. To release the crystal violet, 75% ethanol was added to the wells and OD of stained biofilm was determined by using micro ELISA auto reader at 570 nm. Experiments for each strain were carried out in triplicate and repeated three times.

Exopolysaccharide production assay

For exopolysaccharide determination, flasks (250 mL) including 100 mL of a medium proposed by Verhoef et al. (2003) were supplemented with different NaCl concentrations (0, 50, 100, 150 mM) and inoculated with 24 h old prepared bacterial culture (1000 µl) then incubated at 160 rpm shaker for 48 h at 28°C. To extract exopolysaccharide, the method of De Vuyst et al. (1998) was used. Bacterial cultures were centrifuged at optimized conditions (10000 rpm for 15 min). Supernatant was carefully remove, then wash the cells by 50 mM NaCl, centrifuge at 10000 x g for 5 min and remove supernatant. Repeat washing four additional times. Re-suspend cells in 1 ml of 50 mM EDTA, and incubated at 28°C for 60 min, centrifuged at 14,500 x *g* for 5 min, carefully removed the supernatant and transfer to fresh eppendorf tubes. Exopolysaccharides were quantified in terms of total carbohydrates and measured by the phenol-sulfuric acid method using glucose as a standard (Dubois et al., 1956).

Effect of PGPR biofilm on salt tolerance of faba bean growth

Healthy seeds of faba bean (Nubaria 3 variety) were obtained from Agricultural Research Center, Giza, Egypt. Faba bean seeds were disinfected using 1% sodium hypochlorite solution for 10 min,

Table 2. Physical properties of the experimental soil.

Particle size distribution	(%)
Sand	97.65
Silt	1.51
Clay	0.84
Textural class	Sand
Field capacity %	17.0
pH	7.80

rinsed thrice in sterile distilled. After surface sterilization, seeds were inoculated with selected bacterial strains. Suspension of bacterial cultures (24 h old) was centrifuged for 2 min at 13400 rpm.

Cells were suspended in saline solution (NaCl 1%) to get bacterial suspension (OD 600 nm adjusted 10^8 mL^{-1} CFU). Sterilized seeds were inoculated for 30 minu before sowing. For control, seeds were steeped in sterile water for the same period of time. The PGPR populations were detected by adding suitable amounts of saline solution to obtain 10^8 CFU / 200 ml. These dilutions served as bacterial inoculum for plant experiment, 2.8 kg of winnowed, sterilized and air dried soil was thoroughly mixed with 200 ml diluted bacterial suspension for 2 min. The characters and composition of soil are presented in Table 2.

Seeds were sown in plastic pots for 45 days and irrigated regularly by different concentrations of NaCl (0, 50,100 and 150 mM) per gram weight of soil. After 45 days, the seedlings were harvested and different growth parameters that is, plant length (cm), dry weight (g/plant) and fresh weight (g/plant) were measured.

Statistical analysis

Collected data were statistically analyzed using the appropriate analysis of variance according to Steel and Torrie (1981). The experiment date designated in two ways was completely randomized with three replicates. Computer program software CoStat version 6.311 was used to analyses the data of experiment. Least significant difference (LSD) at 5% level was used separately to evaluate the response of each character.

RESULTS

Out of 20 strains, 12 strains had the ability to produce biofilm. Biofilm producing strains were confirmed by various methods. In the current study, qualitative and quantitative estimation of biofilm production by PGPR strains was performed.

Biofilm qualitative screening

Obtained results observed that thick film was formed inside the wall and bottom of the tube. Twelve strains (SAW 7, SAC1, SAW 10, SAB12, SAW 23, SAW 24, SAB 8, SAW 19, SAW 5, SAW 9, SAW 15 and SAW 1) had thick film inside the wall of the tube denoting that strains could produce biofilm while eight strains could not produce biofilm. The results showed that the purple ring appeared under different levels of NaCl concentrations (0, 50, 100 and 150 mM).

Biofilm quantitative screening

Twelve *Pseudomonas* were assayed for production of biofilm. The results indicated that the activity of biofilm formation was increased with increasing NaCl concentration. The highest significant increase was recorded in seven strains treated with 150 mM NaCl. These antagonistic bacteria were strains (SAW 1, SAB 12, SAW 15, SAW 24, SAW 19, SAW 7 and SAW 9). However, no biofilm ring was formed either in the absence of NaCl in six strains (SAW 24, SAW 1, SAW 5, SAW 9, SAW 23 and SAW 19).

The results in Figure 1 showed the values of absorbance at 570 nm as a measure of optical density (OD) which reflected the activity of biofilm formation of 12 strains in nutrient broth cultures supplemented with four concentrations of NaCl (0, 50, 100 and 150 mM).

Exopolysaccharide production under different salt concentrations

Fxopolysaccharides (EPS) production of PGPR strains under different NaCl concentrations (0, 50, 100 and 150 mM) was tested. The results showed that there was a general direction of gradual increase in EPS with increasing salt concentrations (Figure 1). However, at no salt stress or low concentration of NaCl exopolysaccharide production was reduced (Figure 2).

Effect of inoculation with selected PGPR on plant growth of faba bean

The effect of inoculation with selected PGPR (SAW 1, SAW 24 and SAW 19) on growth of faba bean at four salt concentrations (0, 50, 100 and 150 mM) was studied. The results in Figure 3 showed that the increasing salt stress affect plant height, shoot fresh weight and shoot dry weight. Also, results indicated that the increasing of salinity stress was significantly reduced all growth parameters of non-inoculated plants. Results denoted that in non-inoculated plants, there was a little bit impact on NaCl stress up to 50 mM. The effect of inoculation on dry masses of the salinity stress on faba bean is represented in Figure 3.

PGPR inoculated plants showed higher accumulation of shoot dry mass than their corresponding uninoculated ones under salt stress. However, the inoculation with SAW 24, SAW 1 and SAW 19 gave the highest value in

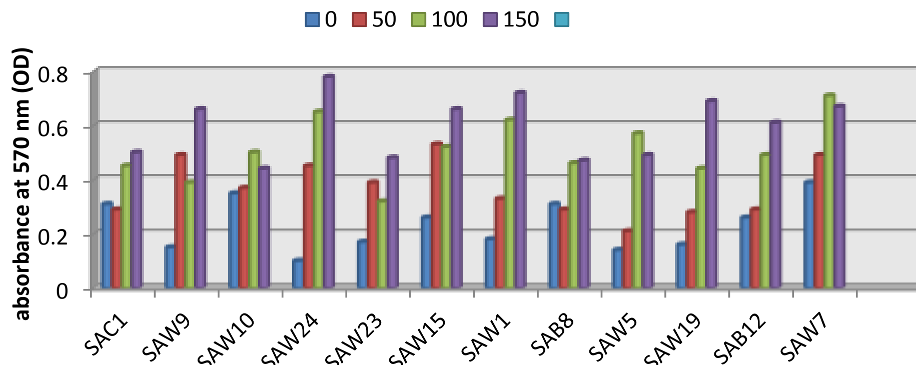

Figure 1. The optical density (OD) (at 570 nm) as a measure of the activity of biofilm formation of 12 strains in nutrient broth cultures supplemented with four concentrations of NaCl (0.0, 50, 100 and 150 mM).

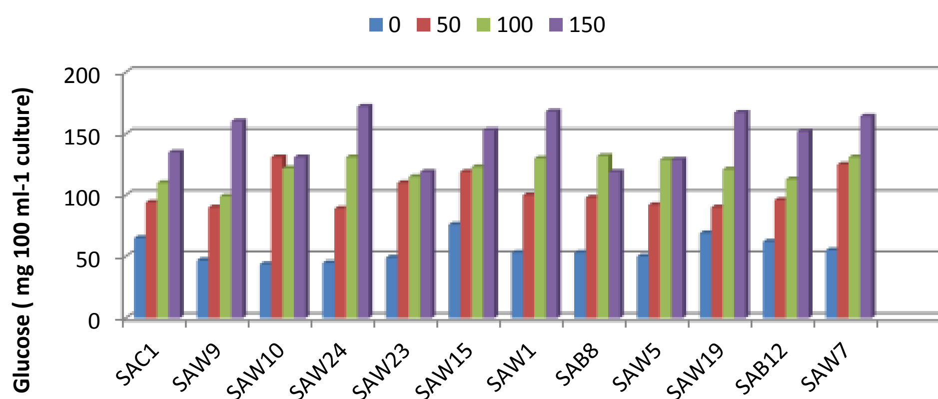

Figure 2. Effect of different concentrations of NaCl (0.0, 50, 100 and 150 mM) on glucose content (mg 100 ml^{-1} culture) of bacterial strains in EPS media.

shoot fresh mass with 7.2, 6.1 and 4.8 g, respectively comparing to 3.6 g in the uninoculated stressed treatment at 100 mM NaCl.

At 100 mM NaCl, the inoculation with SAW 24, SAW1 and SAW 19 PGPR strains enhanced the plant height by 43, 40 and 36%, respectively compared to their uninoculated plants. The PGPR SAW 24 and SAW1 strains enhanced the accumulation of dry mass of shoots by 47 and 42%, respectively at 100 mM NaCl. Maximum increase under stress in the plant height, fresh weight and dry weight of shoot was observed at 50 mM NaCl stress. Depending on obtained results of the present experiment, the results showed that the strain SAW 24 showed the highest activity of biofilm formation under the different levels of NaCl concentrations. Also, the same strain *Pseudomonas anguilliseptica* SAW 24 showed the highest growth measurements of faba bean plants under salt stress.

DISCUSSION

The results show that increasing NaCl concentration leads to the increase in exopolysaccharide production. Moreover, increasing the production of exopolysaccharide against higher salt stress leads to support biofilm formation (Ishii et al., 2004; Fujishige et al., 2006). These results are in agreement with those obtained by Arora et al. (2010), Qurashi and Sabri (2012), Deng et al. (2015) and Kasim et al. (2016).

It was reported that formation of biofilm and exopolysaccharide kept the viability of bacterial cells under salt stress to protect them in the rhizosphere. Previous research showed that biofilm formation and exopolysaccharide production by PGPR strains significantly increase soil fertility and enhance plant growth (Ashraf et al., 2005; Liaqat et al., 2009).

The present results are similar with several previous

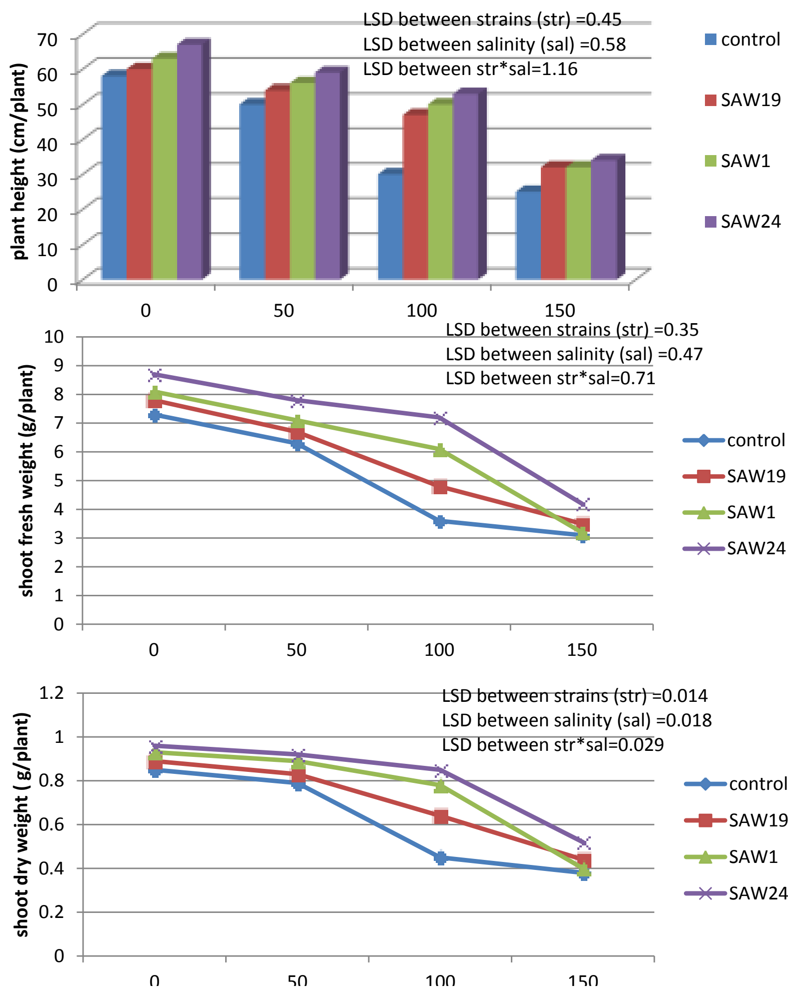

Figure 3. Effect of the three selected PGPR strains biofilm forming and exopolysaccharides producing on plant height, shoot fresh weight and shoot dry weight of faba bean under different salt concentrations.

reports that, salinity stress significantly reduced leguminous crops (Soussi et al., 1998; Chookhampaeng, 2011). However, inoculation with PGPR encouraged the plant growth of *Leucaena esculenta* with and without salt stress by releasing phytohormones which lead to increase bacterial root colonization and biofilm formation (Lugtenberg and Kamilova, 2009; Arora et al., 2010; Ahmed and Shahida, 2010).

Previous studies showed that Bacterial EPS under salt stress can bind sodium ions and reduces its toxicity in the soil (Arora et al., 2010). These may be in the same line with the current study showing the role of EPS to bind Na^+ and reduce its toxic effect. In general, the present results illustrate the effect of PGPR strains on enhancement of faba bean plant growth with and without salinity stress. In addition, the results show that the PGPR strain *P. anguilliseptica* SAW 24 have the highest biofilms formation and exopolysaccharides production under different salt stress. Also, gave the best records of plant height, fresh and dry weight of faba bean plants.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Isolation, identification and bioactivity of fungal endophytes from selected Kenyan medicinal plants

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Infectious diseases remain a global health burden due to development of antibiotic resistance by pathogenic microorganisms. Antibiotic resistance has led to increased number of deaths among children and adults. This study sought to isolate and identify fungal endophytes from selected Kenyan medicinal plants and screen them and their extracts for bioactivity against selected test human pathogens. Fifty-five fungal endophytes were successfully isolated from fresh leaves of twenty-three medicinal plants from Kakamega forest. The DNA of fungal endophytes was extracted and molecular characterization was done through sequencing of the internal transcribed spacer region (ITS). The isolated fungal endophytes belonged to nine genera in the Ascomycota group, namely *Fusarium*, *Colletotrichum*, *Trichothecium, Phomopsis*, *Pestalotiopsis*, *Cladosporium*, *Aspergillus*, *Phoma,* and *Chaetomium*. Extracts from *Aspergillus* sp. demonstrated antimicrobial activity at low concentrations of 2.34 µg/ml against *B. subtilis* and 9.38 µg/ml against *Candida tenius*; while extracts from *Colletotrichum* sp. demonstrated antimicrobial activity at moderate concentration (37.5 µg/ml) against *B. subtilis* in the serial dilution assay. These results show that medicinal plants are a reservoir to a diversity of fungal endophytes that could be exploited as sources of natural products of pharmaceutical importance.

Key words: Antibiotic resistance, antimicrobial, bioactive, fungal endophytes.

INTRODUCTION

Infectious diseases cause about 18.4% deaths worldwide (WHO, 2014) and are linked to the development of multidrug-resistant pathogenic bacteria. Several research initiatives have shown results of drug resistance in human pathogenic bacteria around the world (Laxminarayan et al., 2013). Consequently, attention has been focused on finding alternative antimicrobial compounds from natural sources including endophytes. Fungal endophytes are a group of microorganisms that live in tissues of healthy plants, inter and intra-cellularly

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without causing any disease symptom (Wilson, 1995). Additionally, it is postulated that medicinal plants and endophytes display a mutualistic relationship. In this case, plants act as hosts to these endophytes by providing nutrients and in return, the endophytes produce secondary metabolites, which protect the plants against pathogenic herbivore attack (Yu et al., 2010). Therefore, there is a possibility of fungal endophytes isolated from medicinal plants to produce natural products of pharmaceutical significance.

Medicinal plants growing in natural habitats such as the Kakamega Tropical Rainforest are promising hosts of fungal endophytes that might produce bioactive secondary metabolites of pharmaceutical importance. Additionally, the local community in the treatment of various illnesses uses these plants. Therefore, micro-organisms chosen in this study are a representation of the common pathogens that cause infectious diseases. In this paper, the isolation, identification, and antimicrobial screening of fungal endophytes from Kenyan medicinal plants was reported.

MATERIALS AND METHODS

Isolation of the fungal endophytes

Twenty-three medicinal plants were selected for this study based on available ethno botanical information. Furthermore, fresh and healthy leaves were selected with the help of a plant pathologist. Sampling was done at Kakamega Tropical Rainforest, which lies at 0°10’ to 0°21’N 34°58’ E. Isolation of fungal endophytes was performed by a modification of the method described by Zinniel et al. (2002). In this method, the leaves of the selected plants were washed under running tap water to remove any soil or other foreign materials. Afterwards, they were surface sterilized for three minutes using 70% ethanol followed by soaking in 1% sodium hypochlorite for one minute and rinsed three times with sterile distilled water to remove any traces of the disinfectant. The surface sterilized leaves were then cut aseptically into sections approximately 1 mm by 4 mm and plated in Petri dishes containing potato dextrose agar (PDA) amended with streptomycin sulphate (250 mg/l). The inoculated plates were placed in an incubator at 25 ± 2°C for 1 to 4 weeks. The Petri dishes were monitored after every three days to check for fungal growth. Pure cultures were then prepared by sub-culturing fungal mycelia of each endophyte isolate into sterile Petri dishes containing PDA without antibiotics.

Identification of the fungal endophytes

Identification of the fungal endophytes was done using molecular characterization through sequencing of the internal transcribed spacer region (ITS). However, morphological identification was not possible in this study because the endophytes did not produce fruiting structures in culture.

DNA extraction and amplification

DNA extraction of endophytic fungi was done using BIO BASIC EZ-10 Spin column miniprep kit following the manufacturer’s instructions (Bio Basic Inc.). Fungal endophyte mycelia were obtained from one-week-old cultures and 60 mg of my celium were used for DNA extraction. The internal transcribed spacer (ITS) region of the ribosomal RNA operon was amplified using two primers, ITS1F (CTTGGTCATTTAGAGGAAGTAA) (Gardes and Bruns, 1993) and ITS4 (TCCTCCGCTTATTGATATGC) (White et al., 1990). Polymerase Chain Reaction (PCR) was carried out in a thermal cycler (Applied Biosystems^{TM}) as follows: An initial denaturation of 5 min at 94°C, followed by 35 cycles of denaturation for 3 s at 94°C, 1 min for annealing at 52°C, 1 min for elongation at 72°C and a final elongation of 10 min at 72°C. The quality and quantity of PCR products (3 µL) were checked by electrophoresis on a 0.8% agarose gel stained with Midori green dye and visualization was done with an UV transilluminator (Nippon Genetics Europe GMbH).

Sequencing and phylogenetic analysis

The amplified PCR products were purified according to BioBasic EZ-10 spin column PCR purification kit following manufacturer’s instructions. Sequencing was done using the automated illumina genome analyzer IIX DNA sequencing machine (hiseq). Sequences were compared to ITS sequence data from strains available in the public databases GenBank (http://www.ncbi.nlm.nih.gov/genbank/) by using Basic Local Alignment Search Tool (BLAST) N sequence match routines. The sequences were then aligned using ClustalW software program. Alignments obtained were used to calculate distance matrices and construct phylogenetic trees. Phylogenetic and molecular evolutionary analyses were conducted using Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 (Tamura et al., 2013).

Fermentation and extraction of fungal endophytes

Submerged fermentation was carried out using the method described by Stadler et al. (2001) followed by extraction in acetone and ethyl acetate. The inocula were prepared by introducing 7 mm mycelial agar plugs from 10-day-old fungal culture into 250 mL Erlenmeyer flasks containing 200 mL sterilized sugar malt (SM1/2) and yeast malt (YM6.3) broth medium. The cultures were cultivated at 23°C with a rotational speed of 140 rpm. After 10 to 14 days of incubation, the fungal biomass and the fermentation broth were separated by filtration. The fungal biomass was extracted by soaking in 200 mL acetone followed by mixing on a magnetic stirrer for 5 min and then subjected to an ultrasonic sound bath for 30 min. The fungal biomass was separated from acetone by filtration, then the filtrate which constituted acetone was concentrated by evaporation on a vacuum rotavapor at 40°C and pressure of 540 pa leaving behind the water phase. An equal volume of ethyl acetate was added to the water phase, and the upper organic phase was then concentrated to dryness. The dry extract obtained was weighed and stored at -20°C for further analysis. The final pH of the fermentation broth was measured, followed by extraction with equal volume of ethyl acetate. The upper organic phase was concentrated to dryness on a vacuum rotavapor at 40°C and a pressure of 240 pa. The dry extract obtained was weighed and stored at -20°C for further analysis.

Screening crude extracts of fungal endophytes for antimicrobial activity and minimum inhibitory concentration (MIC) test

Antimicrobial activity of fungal endophyte extracts was determined using the serial dilution assay test as described by Okanya et al. (2011). The test pathogens used were *Bacillus. subtilis* DSM10, *Escherichia. coli* DSM498, *Candida. tenius* MUCL29892, and *Mucor.*

Table 1. Fungal endophyte isolation frequency and their plant sources.

Plant source	No. of endophytes isolated
Piper capense (Wild pepper)	4
Kigelia africana (Sausage tree)	2
Tragia insuavis	3
Teclea nobilis	3
Polyscias fulva (Parasol tree)	2
Afromonum angustifolium	1
Trichilia emetica (Natal mahogany)	4
Markhamia lutea (Nile Tulip)	3
Toddalia asiatica (Orange climber)	2
Clausena anisata	5
Albizia gummifera (Smooth barked flat-crown)	3
Revolva caffra	2
Vernonia amygadalina (Bitter leaf)	1
Tithonia diversifolia	2
Barsamia alba	1
Mondia wheitei (White's ginger)	3
Warburgia ugandensis (Uganda greenheart)	4
Prunus africana (Iron wood)	3
Croton macrostachyus (Broad leaved croton)	2
Zanthoxylum gilletii (African Satinwood)	2
Brugmansia sp.	2
Erythrococca sp.	1
Total	**55**

plumbeus MUCL4935. Overnight 24 h cultures of *B. subtilis* DSM10 and *E. coli* DSM498 were prepared by inoculating 1 ml of the stock cultures in sterile 100 ml EBS media. The cultures were then incubated at 30°C for 24 h. Fungal cultures of *C. tenius* MUCL29892 and *M. plumbeus* MUCL4935 were prepared by inoculating 1 ml of the stock cultures in sterile 100 ml of yeast malt media. Thereafter, the cultures were incubated at 23°C for 48 h. Bacterial cell suspensions were diluted to 10^5 CFU/ml and 280 µl of each cell suspension was pipetted into the first row (A1-A12) of a 96-well plate. In addition, 20 µl of 4.5 mg/ml crude fungal endophyte extracts was pipetted into the first row to make a final concentration of 300 µg/ml. Thereafter, a 2-fold serial dilution with concentrations ranging from 300 to 2.34 µg/ml was made. Cycloheximide and Nystatin were used as reference antibiotic and antifungal respectively with concentrations ranging from 100 to 0.78 µg/ml. The 96-well plates with bacterial pathogens were incubated at 30ºC for 24 h, whereas plates with fungal pathogens were incubated at 23°C for 48 h. The presence of clear wells was used as an indicator of antimicrobial activity. Minimum Inhibitory Concentration (MIC) was determined as the lowest concentration of fungal endophyte extract that inhibited visible growth.

Data analysis

Mean inhibition zones were calculated and equality of means was analyzed using one way analysis of variance (ANOVA).Tukey's Honestly Significant Difference (HSD), a post-hoc analysis, was used to determine if there was any significant difference between the means of the isolates. Data analysis was performed using R statistical software version 3.3.1.

RESULTS

Fifty-five fungal endophytes were successfully isolated from surface sterilized fresh leaves of 23 indigenous medicinal plants sampled in Kakamega Tropical Rainforest, Kenya (Table 1). *Warburgia ugandensis*, *Piper capense*, *Clausena anisata* and *Trichilia emetica* were among the medicinal plants that yielded the highest number of fungal endophyte isolates.

Successful PCR amplification and ITS sequences were obtained for 49 of the isolated fungal endophytes. The ITS PCR products after size separation by agarose gel electrophoresis showed that the PCR products gave the expected DNA band size (500-700 bp) indicating that the primers used successfully amplified the ITS region. The ITS1-5.8S-ITS2 sequences of the isolates were compared to ITS sequences of other organisms represented in the NCBI database GenBank. BLAST results revealed that isolated fungal endophytes belonged to three classes of Ascomycota; Eurotiomycetes, Sordariomycetes and Dothideomycetes. These included nine genera; *Colletotrichum* (44%), *Fusarium* (32%), *Phomopsis* (7%), *Pestalotiopsis* (7%), *Aspergillus* (2%), *Chaetomium* (2%), *Cladosporium* (2%), *Trichothecium* (2%) and *Phoma* (2%) (Figure 1).

Seventeen endophytic fungal isolates (28%) demonstrated antagonism against *S. aureus* ATCC25923

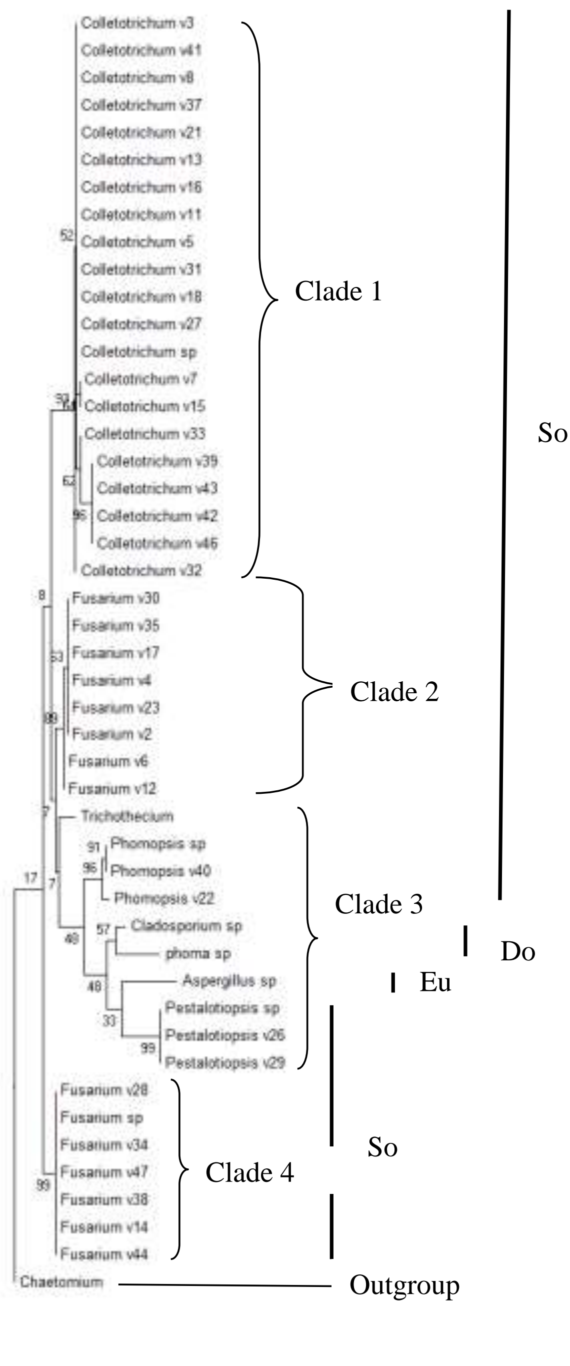

Figure 1. Phylogenetic tree of the isolated fungal endophytes based on ITS analyses (Maximum Likelihood method; 1000 replicate bootstrap). Class: So, Sordariomycetes; Eu, Eurotiomycetes; Do, Dothidiomycetes.

(Table 2). This activity was not significantly different (P< 0.01) from the positive control (chloramphenicol) with inhibition zone >14 mm. Endophytic fungal isolates of the genera *Pestalotiopsis*, *Colletotrichum*, and *Fusarium* were the most active; *Fusarium* sp. (ML1) exhibited a maximum inhibition zone of 17.7±1.20 mm. Besides, antagonism of twenty-two endophytic fungal isolates (36.7%) against *P. aeruginosa* ATCC87853 (Table 2) was not significantly different (P< 0.01) from the positive control. Maximum activity was registered by *Colletotrichum* sp. (PC5) with an inhibition diameter of 17.0±0.58 mm. Isolates of the genera *Phomopsis* sp., *Chaetomium* sp. and *Cladosporium* sp. showed minimal antibacterial activity with significant differences in their

Table 2. Inhibition diameters (mm) ± standard error (se) for the isolates extracts against the test organisms.

Test organism (diameter in mm, n=3)			
Isolate	***S. aureus***	***Isolate***	***P. aeruginosa***
Fusarium sp.(ML1)	17.7±1.20[ab]	*Colletotrichum* sp. (PC5)	17.00±0.58[ab]
Colletotrichum sp. (ZG2)	16.3±1.86[bc]	*Colletotrichum* sp. (RC1)	17.00±1.15[ab]
Fusarium sp. (MW2)	16.00±1.00[cd]	*Fusarium* sp. (BS2)	16.67±0.33[ab]
Colletotrichum sp. (PC4)	16.00±2.30[cd]	*Fusarium* sp. (CM3)	16.33±0.67[bc]
Fusarium sp. (BS2)	15.67±1.20[de]	*Fusarium* sp. (TN3)	16.33±0.33[bc]
Fusarium sp. (CM3)	15.67±1.67[de]	*Colletotrichum* sp. (WU1)	16.33±0.88[bc]
Colletotrichum sp. (PA5)	15.67±0.88[de]	*Trichothecium* sp. (CM2)	15.67±0.88[cd]
Colletotrichum sp. (PC3)	15.67±2.7[de]	*Fusarium* sp. (PA1)	15.67±0.67[cd]
Pestalotiopsis sp. (TD1)	15.67±0.88[de]	*Fusarium* sp. (AA)	15.33±1.20[de]
Fusarium sp. (MW4)	15.33±0.33[de]	*Pestalotiopsis* sp. (BA2)	15.33±0.33[de]
Fusarium sp. (PC1)	15.00±0.58[ef]	*Fusarium* sp.(PC1)	15.33±0.67[de]
Colletotrichum sp. (PC2)	14.67±3.28[fg]	*Colletotrichum* sp. (TI1)	15.33±1.67[de]
Colletotrichum sp. (PC5)	14.67±0.33[fg]	*Fusarium* sp. (ML4)	14.67±0.33[ef]
Pestalotiopsis sp. (TD2)	14.33±2.33[fg]	*Colletotrichum* sp. (PC2)	14.67±0.67[ef]
Colletotrichum sp. (KA1)	14.00±1.73[gh]	*Colletotrichum* sp. (TA2)	14.67±0.88[ef]
Colletotrichum sp. (TA1)	14.00±1.53[gh]	*Fusarium* sp. (BS1)	14.33±0.67[fg]
Fusarium sp. (TN1)	13.67±2.40[gh]	*Colletotrichum* sp. (PA4)	14.33±0.33[fg]
Chloramphenicol	22.5±0.07[a]	*Colletotrichum* sp. (PA5)	14.33±0.88[fg]
Negative control	0.00±0.00[i]	*Colletotrichum* sp. (CA1)	14.00±3.21[gh]
		Colletotrichum sp. (CA3)	14.00±1.00[gh]
		Colletotrichum sp. (PA2)	14.00±1.00[gh]
		Colletotrichum sp. (TN2)	14.00±1.00[gh]
		Chloramphenicol	19.7±0.88[a]
		Negative control	0.00±0.00[i]

Within a column, fungal endophytes sharing the same letter(s) are not significantly different in their inhibition while those with different letters are significantly different (α =0.05, Tukey HSD test) ± Standard error (se).

means from the positive control. Some *Fusarium* sp. (BS1, MW1, and WU2) and *Colletotrichum s*p. (TE4) were inactive against the two bacteria.

Ethyl acetate extracts of fungal endophyte isolates including *Colletotrichum* sp., *Pestalotiopsis* sp. and *Phomopsis* sp. showed antimicrobial activity against *B. subtilis* DSM10, *E. coli* DSM498, *C. tenius* MUCL29892, and *M. plumbeus* MUCL4935. Supernatant extracts of *Pestalotiopsis* sp. isolated from *Tithonia diversifolia* showed antibacterial activity against *B. subtilis* at high concentrations with MIC values of 75 and 150 µg/ml. The extract was not active against *E. coli*, *C. tenius*, and *M. plumbeus*.

Extracts of *Aspergillus* sp. isolated from *Albizia gummifera* was the most effective against all the four test microorganisms. The supernatant extract was active against *B. subtilis* at low concentrations with MIC value of 9.38 µg/ml in YM6.3 media and a value of 2.34 µg/ml in SM1/2 media. Similarly, mycelial extract of the same isolate against *C. tenius* recorded MIC value of 9.38 µg/ml. In contrast, the antibacterial activity of the same extracts against *E. coli* was demonstrated at high concentrations of 75 µg/ml (Table 3). The same case was observed with antifungal activity at high concentrations of150 µg/ml against *M. plumbeus*.

Supernatant extracts in SM1/2 media of *Colletotrichum* sp. isolated from *Kigelia africana* demonstrated antibacterial activity against *B. subtilis* at a high concentration of 300 µg/ml. In contrast, supernatant extracts from YM6.3 media were active against *B. subtilis* at much lower concentration, recording MIC value of 37.5 µg/ml. However, extracts of these isolates were not active against *E. coli*, *C. tenius*, and *M. plumbeus*. Extracts of *Phomopsis* sp. isolated from *Trichilia emetica* were active against both *B. subtilis* and *M. plumbeus* at high concentrations of 75 and 150 µg/ml (YM6.3 media) and 150 µg/ml (SM1/2 media) respectively.

DISCUSSION

Currently, molecular identification techniques stand out as the most effective method for identification of non-sporulating fungal endophytes and the detection of viable but non-culturable fungi (Grunig et al., 2002). Therefore, fungal endophytes in this study were subjected to

Table 3. Minimum inhibition concentration (MIC) values of fungal endophyte extracts against selected test bacteria and fungi.

				MIC values (µg/mL)			
Isolate	**Endophyte**	**Media**	**E**	*B. subtilis*	*E. coli*	*M. plumbeus*	*C. tenius*
TD1	*Pestalotiopsis* sp.	YM 6.3	S	150	*	*	*
			M	*	*	*	*
		SM1/2	S	75	*	*	*
			M	*	*	*	*
KA1	*Colletotrichum* sp.	YM 6.3	S	37.5	*	*	*
			M	*	*	*	*
		SM1/2	S	300	*	*	*
			M	*	*	*	*
AG2	*Aspergillus* sp.	YM 6.3	S	9.38	75	*	150
			M	2.34	*	*	*
		SM1/2	S	2.34	18.75	150	75
			M	2.34	75	*	9.38
TE5	*Phomopsis* sp.	YM 6.3	S	75	*	150	*
			M	150	*	*	*
		SM1/2	S	150	*	*	*
			M	150	*	*	*
TE2	*Phomopsis* sp.	YM 6.3	S	*	*	*	*
			M	150	*	*	*
		SM1/2	S	*	*	*	*
			M	300	*	*	*
KA2	*Phomopsis* sp.	YM 6.3	S	150	*	*	150
			M	*	*	*	*
		SM1/2	S	*	*	*	300
			M	*	*	*	*

E, Extracts; S, Supernatant; M, Mycelia; *, No activity; SM, Sugar malt media; YM, Yeast malt media.

molecular identification based on sequencing the ITS1-5.8S-ITS2 region. The latter generates a considerable sequence variation between closely related species due to a faster rate of evolution thus, sequences of these regions provide a good resolution at lower taxonomic levels (genus and species level). Use of ITS sequences, however, have shortcomings in that they might not achieve a perfect sequence alignment at higher taxonomic levels such as family, order and class (Lindahl et al., 2013) due to high ITS variability. Also, about 20 to 30% of ITS sequences obtained from GenBank and other public databases for comparative analysis may not be accurate in their identification (Huang et al., 2009), a challenge that was equally encountered in this study as well. The drawback is attributed to the fact that most of the sequences deposited in these public databases fail to match the organism in question. Moreover, previous studies have indicated that the little ITS variation in ascomycete fungi makes the region undesirable for taxonomic identification at the species level (Jang et al., 2014). Similar to this study, isolated fungal endophytes were classified under the phylum Ascomycota whose identification was only achieved at the genus level.

In this study, DNA extraction and PCR amplification were successful; only about 4% of the fungal endophyte isolates were not amplified. Lack of PCR amplification could be due to some of the very likely reasons such as the bias of primers which may amplify ITS regions towards certain groups of fungi (Bellemain et al., 2010). Based on our results, ITS1F and ITS4 were amplified more efficiently in the three classes from the phylum Ascomycota. Some ITS primers like ITS1 and ITS5 are usually biased towards the amplification of Basidiomycetes while others, such as ITS2, ITS3, and ITS4 lean towards Ascomycetes (Bellemain et al., 2010). Another reason could be probably due to the production of PCR inhibitory secondary metabolites in culture (Paterson, 2004). Forty-nine PCR products were subjected to DNA sequencing, which yielded 47 readable sequences that were compared with other sequences in the NCBI database for identification. Two PCR products had no readable sequences; this could be due to errors

that might have occurred during the sequencing process considering that the process is sensitive. Another reason might be weak PCR products obtained during amplification.

BLAST analysis revealed high percentage similarity of different species among the same genera, suggesting that ITS region of some isolates is conserved. A phylogenetic tree was inferred based on the DNA sequences obtained. Fungal endophytes in the genera *Pestalotiopsis* (99%), *Phomopsis* (96%) and *Fusarium* (99%) exhibited high bootstrap values, whereas *Aspergillus* (33%) and *Chaetomium* (17%) showed low bootstrap values. Bootstrap values represent phylogenetic accuracy (Felsenstein, 1985); bootstrapping test whether the dataset supports the generated tree. Values above 50% are likely to indicate reliable groupings (Hillis and Bull, 1993) while lower values mean that the node in question was found in less than half of the bootstrap replicates. Concerning the findings obtained in the current study, it can be deduced that fungal endophytes belonging to *Fusarium*, *Colletotrichum*, *Pestalotiopsis*, *Phomopsis*, *Cladosporium*, and *Phoma* were reliably grouped (bootstrap values > 50%) as opposed to *Chaetomium* and *Aspergillus* (bootstrap values < 50%).

A majority of the isolates showed sequence homology to the genera *Colletotrichum* and *Fusarium* while a few sequences exhibited sequence homology with the genera Pestalotiopsis, *Phomopsis*, *Phoma*, *Cladosporium*, *Aspergillus*, and *Chaetomium*. Although a definitive taxonomic identification (at species level) of fungal endophytes isolated in this study was not achieved, the data generated indicate a great diversity of these organisms in the medicinal plants. These results, therefore, show that molecular markers are an essential part of the phylogenetic analysis, and it may lead to further research on endophytes, their characterization, and assessment of their genetic diversity. Further research should focus on definitive taxonomic identification methods using molecular markers such as β-tubulin gene and elongation factor 1α gene (EF-1α) in addition to the ITS rDNA gene in molecular identification of the isolates to the species level.

The fungal endophytes isolated in this study belong to commonly isolated taxa similar to *Colletotrichum*, *Pestalotiopsis*, *Phomopsis*, *Cladosporium*, *Chaetomium*, *Aspergillus* and *Fusarium*, which have been previously reported as endophytes in medicinal plants (Rakotoniriana et al., 2008). Most of the fungal endophytes such as those from the genera *Aspergillus*, *Colletotrichum*, *Pestalotiopsis*, and *Phomopsis* isolated in this study showed potential antimicrobial activity against test pathogens. Similarly, other studies have reported antimicrobial activity of extracts from these endophytes against pathogenic bacteria (Zhang et al., 2009; Chapla et al., 2014). Usually, in addition to plants producing their secondary metabolites, endophytes equally contribute to the production of secondary metabolites in plants. Secondary metabolite production by endophytes is primarily linked to reducing competition from other microorganisms within the host plants. Further, the production of these natural products defends both the host plant and endophytes from disease-causing microorganisms (Ludwig-Müller, 2015). The bioactivity displayed by extracts from the fungal endophytes in this study could contribute to the medicinal properties of the medicinal plants.

Extracts of *Aspergillus* sp. were the most effective against the test microorganisms with MIC values ranging between 9.38 µg and 2.34/ml. *Aspergillus* sp. has been reported to be major producers of mycotoxins harmful to humans. In the current study, *Aspergillus* sp. isolates exhibited the maximum antimicrobial activity against the test bacteria and fungi. There have been reports of antimicrobial activity of extracts from *Aspergillus* sp. For instance, compounds classified as tremorgenic mycotoxins were identified from the endophytic fungus *Aspergillus* sp. Some of these compounds were reported to have potent antibacterial activity against *E. coli* (Qiao et al., 2010). Other studies have documented bioactive secondary metabolites with structures similar to Ochratoxin A moiety, one of the most abundant mycotoxins found in food (Moore et al., 1972). Further, Traditionally, *A. gummifera*, *K. africana*, and *T. emetica* have been used in the treatment of ailments including stomachache, malaria, and dermatitis. Regarding these reports, this could mean that the bioactivity demonstrated by this fungal endophyte in this current study, could be due to the high production of mycotoxins as secondary metabolites.

Conclusion

From the results of this study, it can be concluded that medicinal plants are a host to a diverse group of fungal endophytes with varying degree of antimicrobial activity. An *Aspergillus* sp. isolates demonstrated a spiking antimicrobial effect against the test organisms. Besides, secondary metabolites produced by *Colletotrichum* sp., *Fusarium* sp., *Pestalotiopsis* sp. and *Phomopsis* sp. isolates also showed antimicrobial activity against the test microorganisms. The bioactive endophytes should be identified to species level. Besides, the bioactive compounds produced by these endophytes should be further characterized for future study inference.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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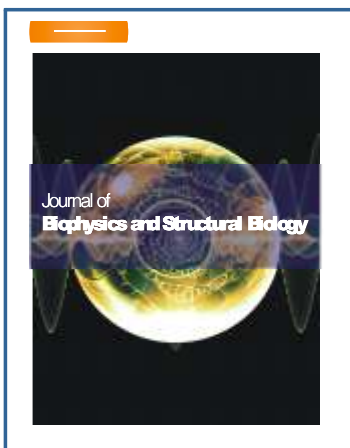

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