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African Journal of Microbiology Research

Review

Ruminal microorganism consideration and protein used in the metabolism of the ruminants: A review

Tiago Neves Pereira Valente[1]*, Erico da Silva Lima[2], Wallacy Barbacena Rosa dos Santos[3], Andréia Santos Cesário[3], Cássio Jardim Tavares[1], Ítalo Lacerda Fernandes[1] and Marco Antônio Moreira de Freitas[1]

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A great diversity of species of microorganisms are present in the rumen environmental with specific functions in the degradation of carbohydrates, protein and lipids. However, the knowledge of the interactions between the different species of microorganisms in the rumen ecosystem and their specific substrates were used to improve nutritional management and can increase production of meat or milk. A balanced nutritional management is very important. When inappropriate feedstuffs are used on diet formulation for cattle, there is a decrease in the growth of microorganisms in the rumen. And the availability of the use of protein synthesized in rumen for all metabolisms of the animal.

Key words: Microorganism growth, microbial protein, proteolysis.

INTRODUCTION

The rumen is a suitable environment for the development of a large number of anaerobic microorganisms, having unique characteristics such as temperature around 38 to 42°C (Pourazad et al., 2015). But normally, the temperature was more commonly found to be 39°C (Hoover and Miller, 1991; Kim et al., 2014; Yazdi et al., 2015). In the rumen, a redox potential was found (-350 mV, with fluctuations between -250 and -450 mV), with result of a strong ambient environment due to the lack of oxygen. The rumen is usually well buffered, due to the presence of bicarbonates and phosphates founded in continuous flow of saliva (Puggaard et al., 2011; Røjen and Kristensen, 2012; Storm et al., 2014). Saliva production can be high in a cow, reaching over 180 L/day. However, the pH can vary due the nature of the diet, but typically found between 5.5 and 7.0 when ruminants are fed with predominantly on forage diet (Aschenbach et al., 2014). According to Pourazad et al. (2015), highly fermentable diets are rapidly converted to volatile fatty acid (VFA) in the rumen. The resulting release of protons can constitute a challenge to the ruminal ecosystem and animal health. Although increased acid production is a nutritionally desired effect of increased concentrate feeding, the accumulation of

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protons in the rumen is not. Because pH values below 6.0 can reduce the survival of bacteria degrading cellulose and synthesis protein. Similar results were observed by Russell and Strobel (1987). Rumen fermentation appeared to continue unimpeded, but when the mean rumen pH was 5.4 it resulted in reduced fiber digestion and less microbial protein flowing to the small intestine (Hills et al., 2015). In the gas phase, carbon dioxide and methane were gases that are more present. Oxygen varies from less than 0.1% depending on the amount of water and feed ingested. The osmotic pressure of rumen contents ranges between 260 and 340 moles with an average value of about 280 moles. Values greater than 350 can cause stop rumination process and a decrease in the absorption of VFA (Russell, 2002).

RUMEN MICROBIAL DIVERSITY

The ruminal microorganisms are composed mainly by three groups of anaerobes; by bacteria, protozoa and fungi. The populations of each group and their species are directly influenced by the type of feedstuff provided to the ruminant host. The rumen-reticulum represents more than 50% of the digestive capacity, consisting 10^{10} bacteria, 10^6 protozoa and 10^4 fungi per milliliter of rumen content (Theodorou and France, 2005; Krause et al., 2014). The microbial population has three important nutritional functions for animals: (1) Digestion and fermentation of carbohydrates, such as cellulose and starch, with consequent production of VFA. (2) Amino acid (AA) synthesis from non-protein nitrogen (NPN) from the diet or from recycling via saliva or ruminal and diffusion from dietary rumen degradable protein. (3) The synthesis of B-complex vitamins and vitamin K. Microbial populations can be subdivided into those free in liquid phase, those linked to food particles, and those attached to the ruminal epithelium (Cheng and McAllister, 1997).

Bacteria

The classification of ruminal bacteria may vary depending on the substrate offered the animal, according to Koslozki (2002), as follows:

(1) Fermenters of structural carbohydrates: Degrade cell wall components of plants, especially cellulose and hemicelluloses, have a relatively slow growth rate and depend on ammonia and branched-chain fatty acids for the synthesis of proteins (isovalerate, isobutyrate and 2-metilbutyrate), for example, *Ruminococcus albus* (Suen et al., 2011), *Fibrobacter succinogenes, Ruminococcus flavefaciens*.

(2) Fermenters of non-structural carbohydrates (CNE): Associate the particles of cereal grains or granules of starch and can use ammonia, AA or peptides (PEP) for synthesis of proteins. As we have increased our knowledge to modify the microbial population to enhance production efficiency and animal health. Because lactic acidosis is an animal health issue related to rumen function, it became an early target for fermentation modifications (including the use of ionophores) (Krause et al., 2014). Much of the development of lactic acidosis (acute, subacute, and chronic) has been linked to increasing populations of *Lactobacillus* species, *Ruminobacter amylophilus* and *Streptococcus bovis*, after increased availability of fermentable soluble starch in the rumen (Plaizier et al., 2012).

(3) Proteolytic: Most of the ruminal bacterial species degrade proteins. However, there are a few species that have a proteolytic activity far more intense than the others, for example, *Peptostreptococus* species; higher *Prevotella ruminicola* and *Streptococcus bovis*.

(4) Methanogenics: Are the most strictly anaerobic rumen. Produce methane from CO_2 and H_2 fermentative activity derived from other species. Methane (CH_4) emissions from ruminant livestock are 90% made from enteric fermentation. Reduction of carbon dioxide to CH_4 is critical for efficient ruminal fermentation, because it prevents the accumulation of reducing equivalents in the rumen (McAllister et al., 2015). The methanogenics are members of the Kingdom Archae (Russell, 2002), for examples: *Methanobacterium* species and *Methanobrevibacter* species. The relationship between methanogens and protozoa is expected. Studies have shown that reducing protozoa populations would reduce CH_4 production indirectly (Leahy et al., 2010; Hook et al., 2010; Wright and Klieve, 2011; Attwood et al., 2011). Second McAllister et al. (2015) methanogens exist in a symbiotic relationship with rumen protozoa and fungi and within biofilms associated with feed and the rumen wall. Studies of the ecology of ruminal methanogenesis are important in ruminant nutrition and identifying ways for its mitigation. According to Hünerberg et al. (2015), several factors such as fast passage rate or cattle fed high-grain diets can decrease CH_4 emissions. However, lowering ruminal pH alone is, therefore, not an effective CH_4 mitigation strategy. Other examples of CH_4 mitigation occur when add lipid in the ration of ruminants, methane emission would reduce by 2.2 to 5.6% (Beauchemin et al., 2008; Martin et al., 2010).

(5) Lactic: Growing under conditions of low rumen pH and using, among others, lactic acid as an energy substrate. Marx et al. (2011), for example: *Megasphaera elsdenii* is a lactate-utilizing bacterium whose ruminal abundance has been shown to be greatly elevated during milk fat depression. Second Weimer et al. (2015) studied the effects of the addition of *M. elsdenii* and concluded milk yield and composition were not affected by dosing. Ruminal pH, VFA and lactate did not differ between dosed and control cows, although acetate-to-propionate ratio declined in both groups and butyrate increased after dosing with *M. elsdenii*. The results confirm that

establishing exogenously added bacterial strains in the rumen is difficult, even for strains previously isolated.
(6) Pectinolitics: Ferment pectin. Although pectin is a polymer of a structural nature, its fermentation, as well as the characteristics of bacteria that use it, are similar to those that ferment the non-structural carbohydrates, for example, *Succinivibrio dextrinosolvens* and *Lachnospira multiparus*.
(7) Lipolytic: Hydrolyze triglycerides into glycerol and fatty acids such as *Anaerovibrio lipolytica*; despite the importance of understanding lipolysis and its impact on subsequent biohydrogenation of polyunsaturated fatty acids by rumen microbes. According to Prive et al. (2013), the enzymes had higher activities at neutral to alkaline pH and had higher hydrolytic activity against caprylate (C8:0), laurate (C12:0), and myristate (C14:0).
(8) Ureolitics: Adhered to the ruminal epithelium, hydrolyze urea and release ammonia in the rumen, as *Enterococcus faecium* (Khattab and Ebeid, 2014).

Protozoa

Protozoa can contribute with more than half of the rumen microbial mass (Van Soest, 1994). However, the flow out of the rumen protozoa is not proportional to its number in the rumen. Protozoa are retained because of biomass selectively in the rumen (Williams et al., 2008). The removed of rumen protozoa increases the flow of microbial and dietary protein to duodenum. In animals feed diets of low nutritional value and high in fiber, evidence the increase in the number of protozoa. Some protozoa are cellulolytics. The main sources of energy supply to the protozoa are carbohydrates, with preference for the use of starch and sugars, compared to diet high level of fiber. The main source of nitrogen (N) is the protein feedstuff, or microbial available through engulfment of bacteria in rumen environment. Protozoa use insoluble proteins rather than soluble proteins, while bacteria did not use ammonia for AA synthesis (Dijkstra et al., 1998).

Many types of protozoa in rumen have different participations on ruminal fermentation, some being beneficial to ruminants and not others (Williams et al., 1991; Williams and Coleman, 1997). *In vivo* experiments have established that mixtures of protozoa ciliates decrease the flow of ammonia N of the stomach into the intestine (Ivan et al., 2000). Despite the greater flow of N ammoniac in animals without protozoa (named defaunated), compared with the non-removed rumen protozoa, second Veira (1986), most of the experiments showed a lower growth rate due to the elimination of rumen ciliates protozoa. Defaunation decreased NH_3-N concentration and increased the conversion efficiency of blood urea N and protein-derived NH_3-N conversion into microbial protein in the rumen. According to Firkins et al. (2007) more studies should be done for characterization of protozoa interactions with proteolytic and deaminating bacterial populations. For example, dairy cattle have greater intakes of readily available carbohydrate combined with increased ruminal passage rates, decreased protozoa biomass relative to bacterial biomass and increase the efficiency of protozoa growth. Thus, reducing the negative effects of bacterial predation, compared with the beneficial effects that protozoa have on stabilizing the entire microbial ecosystem. The addition of lipid in the ration of ruminants negatively affects protozoa population (Szumacher-Strabel et al., 2004; Varadyova et al., 2007; Szumacher-Strabel and Cieslak, 2012).

Belanche et al. (2015) study the effect of diet and absence or presence of rumen protozoa on the rumen microbial community in lambs. In studying the presence of protozoa buffered the effect of diet on the rumen bacterial population. Faunated animals fed alfalfa hay had a greater abundance of *F. succinogenes*, anaerobic fungi and methanogens, as well as an enhanced rumen bacterial diversity. Cellulolytic bacteria were more abundant in solid-associated bacterial fractions.

Fungi

The anaerobic fungi are part of the natural micro-organisms of the rumen and its occurrence is known since the 1970s (Orpin and Joblin, 1997). The 3 species of anaerobic fungi, second Krause et al. (2014), *Neocallimastix frontalis, Sphaeromonas* (now named *Caecomyces*) *communis,* and *Piromonas* (now named *Piromyces*) *communis.* Fungi degrade cellulose more efficiently than the main species of ruminal cellulolytics bacteria. There is evidence that the ruminal inoculation with fungi may improve digestion and fiber ingestion (Grenet and Barry, 1988), because fungi are capable to digest cellulose and hemicelluloses, even when these carbohydrates are present in lignified cell walls. The greater ability of fungi in relation to bacteria is in breaking the cell wall of plants. Therefore, it is comprehensible that the greater participation of the roughage in the diet of ruminants, the greater the contribution of the fungus in the digestive process. Second Faichney et al. (1997) in relation with N available made anaerobic fungi contributed only 11 to 35 g N/kg of the microbial-N in the rumen and 7 to 27 g N/kg of the microbial-N flowing to the duodenum.

TECHNIQUES FOR STUDIES OF FEEDSTUFF DEGRADATION BY RUMINAL MICROORGANISMS

The evaluation of digestion parameters in ruminant animals is assumed for techniques *in situ* or *in vitro* (Broderick and Cochran, 2000; Krizsan et al., 2013; Ramin et al., 2013). Among these techniques, the *in situ*

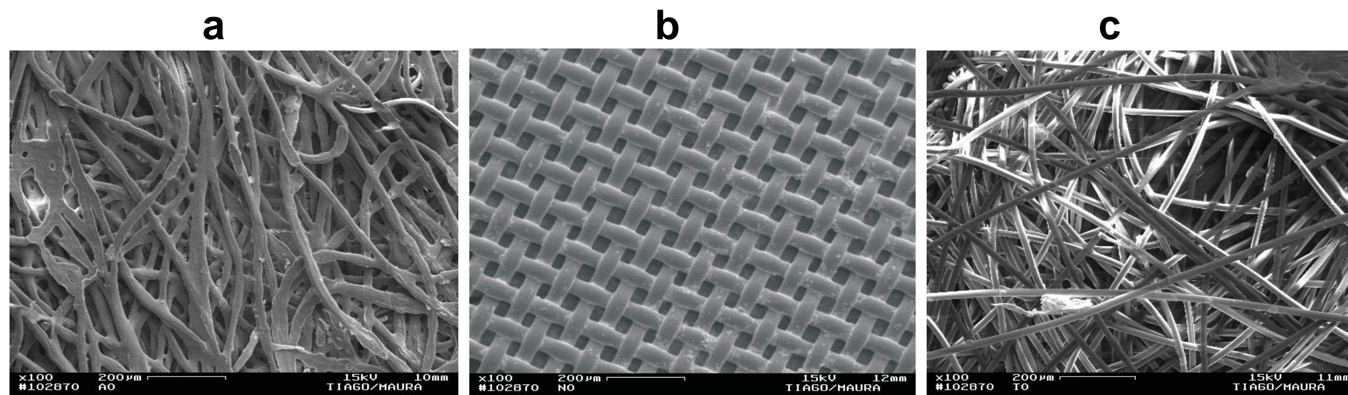

Figure 1. Photomicrographs (X 100) of F57 textile (a), of nylon textile (b) and NWT (c) (Valente et al., 2011b).

evaluations have been more recommended, because the measurements are performed in the rumen, where the degradation process is supposed to be more reliable than *in vitro* (Zhou et al., 2012). In addition, the *in situ* evaluation avoids the accumulation of final products of fermentation, which can affect degradation as sometimes observed under *in vitro* environments. On the other hand, the mathematical modeling of the *in situ* degradation profiles allows the estimation of different parameters of the rumen dynamics, including rate and extension of degradation, the effectively degraded fraction by ruminal microorganisms. Different textiles have been suggested to make the bags used in the *in situ* evaluations, such as nylon (50 µm), F57 (Ankom®) and non-woven textile (NWT, 100 g/m^2) (Casali et al., 2009; Valente et al., 2011a, b). In order to consider a textile useful for ruminal incubation, bags must present porosity thin enough to avoid loss of intact or non-degraded particles and be wide enough to allow the inflow of rumen fluid and microorganisms and the outflow of degradation products and to assure that microbial activity inside bags is similar to that observed in the ruminal environment (Valente et al., 2015). Analyze photomicrographs zoom (X 100) can be observed in Figure 1.

The geometrical structures are similar for F57 and NWT and differ of nylon bag arrangement. However, the objective of both bags is not to avoid the inflow of microorganisms and the outflow of final products of fermentation, important for knowledge, and the ruminal degradation profile by microorganisms in rumen studies (Valente et al., 2015).

NITROGENOUS COMPOUNDS METABOLISM IN THE RUMEN

The quality of ingested protein by ruminants and their AA profile may be advantageous or not for the ruminal microorganism. Ruminal bacteria can use and transform low quality proteins into high quality proteins for the cattle (microbial proteins). However, proteolysis activities were beneficial to the ruminal bacteria during the fermentation process for producing proteins of high biological value (Mackie and White, 1990; Rodríguez et al., 2007).

Ruminal bacteria are responsible for converting about 60 to 90% of N consumed by cattle in ammonia source. Studies indicated that about 50 to 70% of N from bacterial origin can be derived from ammonia (Kosloski, 2002). Using ammonia marked ^{15}N, reveal the relative importance of the N sources of microbial origin and depending on the animal diet (Nolan and Dobos, 2005) found results in 40, the 95% of N of microbial origin from the ammonia dietetics, after turnover rate recycle in rumen environmental. Many bacteria can use ammonia as a source of N for their development, but the concentrations of ammonia in the rumen present considerable fluctuations (Figueiras et al., 2015). Recent research showed that high concentrations of ammonia in the rumen liquid can result from a decrease in transport of urea from the blood to the rumen (Bannink and Tamminga, 2005). Second Abdoun et al. (2007) independent spend the energy urea throughout epithelium recycling because of movement of blood urea.

The concentration of urea in the visceral port system is always less than the arterial. This N was used in the synthesis of microbial protein and urea circulated through the gastrointestinal tract. But Lindsay and Reynolds (2005) note that the microbial protein formed in the large intestine possibly be lost in the feces, although urea reaching the rumen and may be used for protein formation or simply pass and be absorbed in the small intestine. Bacteria require at least 1% of N for its maintenance, and the urea recycling can occur either via saliva or by diffusion through the rumen wall (Van Soest, 1994).

According to Russell et al. (1992), ammonia in excess may be lost as urea by animal urine. However, diets containing low degraded protein in the rumen (RDP), ammonia concentration can be much smaller. The ruminant retains urea in body, avoid urea loss, mainly

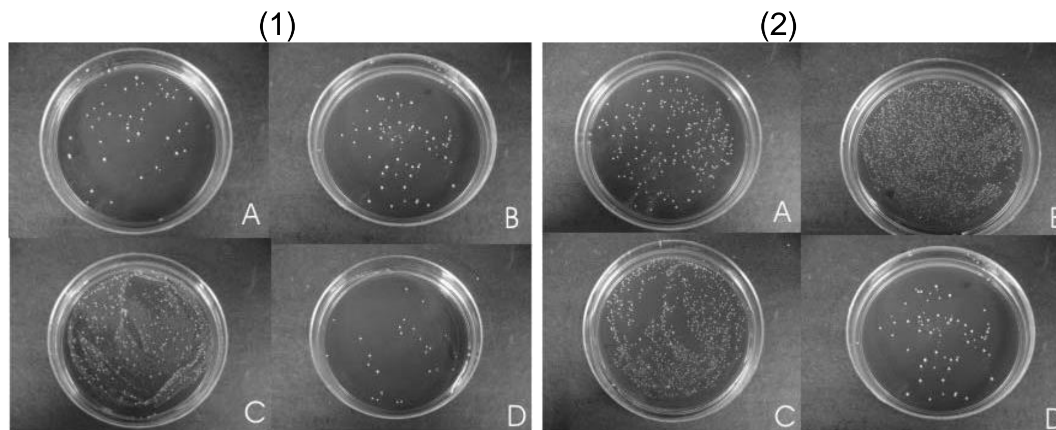

Figure 2. Lactic acid bacteria growth on cellulose (1) on starch (2) with different nitrogen sources after 24 hours (A - control; B - casein; C - soy peptone; and D - urea. The fluid was X 100 diluted). (photo: Carvalho et al., 2011).

through the kidneys. With low protein urea diets, various mechanisms seem to reduce renal excretion of urea, being redirected to the ruminal environment, when the availability of the RDP is low, this can limit microbial growth (Obara et al., 1991; Marini and Van Amburgh, 2003).

Increase in development of microorganisms in the rumen occurs when supplementation with N are realized (Figueiras et al., 2010; Costa et al., 2011). Satter and Slyter (1974) initially studied levels of microbial protein production and concluded that the levels of production were constant with levels of 13 to 14% of CP in the diet. However, the variations in turnover and in rates of fermentation can cause variation in microbial production. And after study, inside the rumen N can be absorbed through the epithelium and the assimilation of ammonia is typically mediated by glutamate dehydrogenase (GDH) or glutamine synthetase (GS). The assimilation of ammonia by GDH is advantageous when the concentration of ammonia is high and carbohydrates (ATP) are limiting. While the GS way is only advantageous when the concentration of ammonia is low and ATP of carbohydrates is not the limiting factor (Russell, 2002).

INFLUENCE OF TYPE OF SUPPLEMENTATION ON RUMEN DEGRADATION

In experiment with cattle, Bailey et al. (2012) observed the effects of supplemental energy sources on nutrient digestion. Supplementation with glucose tended to decrease ruminal neutral detergent fiber (NDF) digestibility. Otherwise, more precision of the rate of increasing casein supplementation increased ruminal concentrations of NH_3, acetate, and propionate. Supplemental energy decreased plasma urea-N concentration, but casein level did not affect it.

In a study with cattle to evaluate the effects of supplementation with different sources of energy and nitrogenous compounds on the interaction of the *in vitro* growth and production of bacteriocin of lactic acid bacteria Carvalho et al. (2011), selected microorganisms according to the energy sources and nitrogenous compounds and concluded that starch promote growth of lactic acid bacteria when compared to cellulose. Supplementation with true protein (soy peptone and casein) stimulated the growth of these bacteria when compared to without supplementation with nitrogenous compounds. Moreover, urea addition does not stimulate the growth of lactic acid bacteria. Sources of true protein increase the competition between non-structural and structural carbohydrates fermenting bacteria (Figure 2).

MICROBIAL PROTEIN CONSIDERATIONS

The average composition of ruminal bacteria is approximately 62.5% crude protein (CP), 21.1% carbohydrates, 12% fat and 4.4% ash at the base of the dry matter (DM) (Russell et al., 1992). According to Russell (2002), increasing the production and flow of rumen microbial protein to the gastrointestinal tract are necessary to maximize livestock production and to reduce their dietary protein requirements. Most of the AA absorbed by ruminants are derived from microbial protein synthesized in the rumen. Dietary metabolizable protein requirements are met by absorption in the small intestine thought microbial protein or dietary protein is not degraded in the rumen (RUP). In ruminant nutrition, maximizing the microbial protein flow to the small intestine, allows in most cases to increase production efficiency. Indeed, utilization of formulated diets, rich in RUP, do not allow optimal growth for animals, because of their deficiency in lysine and methionine (Ali et al., 2009).

Table 1. Fermentation products (mmol l^{-1}) of hyper-ammonia-producing bacterial isolates* obtained from Nellore steers.

Concentration of fermentation end-products	Isolate Gram +	Total VFA (mmol l^{-1}**)	A	P	B	Ib (%)	F	S	Iv
High	C48	79.9[a]	47.8	8.1	7.8	5.3	4.37	4.8	21.5
	C51	76.6[a]	54.2	7.1	7.3	5.1	1.57	1.5	23.0
	C33	73.3[a]	55.4	7.9	5.8	5.9	2.37	nd	22.3
	R34	71.0[a]	37.4	3.7	5.9	5.3	1.78	1.7	44.0
	C11	68.4[a]	69.1	11.8	6.2	7.2	1.32	nd	4.2
	R36	66.7[a]	50.3	11.3	5.1	5.1	1.48	nd	26.5
	C47	64.4[a]	49.2	8.4	7.7	5.8	1.95	nd	26.7
	C37	62.5[a]	65.9	13.9	6.8	6.5	1.69	nd	5.0
	R40	60.6[a]	37.8	10.2	5.5	4.0	9.48	nd	32.9
	R90	60.2[a]	64.9	9.7	5.4	9.7	3.63	1.5	4.9
	R15	59.6[a]	67.4	10.1	6.9	8.2	1.94	0.5	4.7
	R23	53.7[a]	61.8	12.0	8.3	9.5	5.14	nd	3.0
Medium	C89	49.2[b]	57.1	9.5	7.3	6.0	14.9	2.5	2.4
	C34	45.5[b]	63.3	14.6	7.2	7.2	3.00	nd	4.5
	C122	45.0[b]	57.9	10.4	7.3	5.6	13.44	2.9	2.2
	R50	44.2[b]	66.8	10.1	7.3	7.8	2.89	nd	4.9
	R61	44.0[b]	39.0	7.3	6.6	4.4	Nd	nd	42.5
	C114	43.6[b]	51.4	10.4	6.5	7.8	21.72	nd	2.0
	C118	41.6[b]	55.7	10.1	7.4	7.9	12.63	3.2	2.6
	R51	39.0[b]	50.1	16.9	9.0	6.8	13.43	1.6	1.9
	C54	38.3[b]	51.5	16.5	8.8	8.3	12.86	nd	1.8
	C117	34.5[b]	49.4	15.0	2.6	13.6	8.69	6.3	4.1
Low	R21	30.6[c]	71.5	9.0	8.7	5.9	Nd	nd	4.7
	R60	30.3[c]	66.0	8.4	13.5	6.2	Nd	nd	5.8
	R91	25.0[c]	20.2	10.2	3.5	56.2	8.02	nd	1.6
	R63	22.9[c]	64.7	11.6	10.3	7.3	Nd	nd	5.9
	R107	21.5[c]	58.8	15.4	3.6	13.5	6.59	nd	1.9
	C116	19.3[c]	50.0	17.2	nd	15.4	10.61	6.6	nd
	R96	16.9[c]	52.2	17.3	4.5	11.6	11.13	nd	3.0
	R97	12.2[c]	47.8	25.7	nd	7.3	19.05	nd	nd

VFA: Volatile fatty acid; A: acetate; P: propionate; B: butyrate; Ib: isobutyrate; F: formate; S: succinate; Iv: isovalerate; nd : not detected. Values are provided as the mean ± standard deviation of the mean. *The isolates were grown in anaerobic mineral medium supplemented with 15 g l^{-1} trypticase for 24 h at 39°C. **Averages of total volatile fatty acids followed by different letters in the same column differ at 5% probability by the Scott-Knott test (Bento et al., 2015).

PROTEOLYSIS IN THE RUMINAL ENVIRONMENT

Understand of characteristics of the ruminal microbial population has opened new avenues of microbial ecology, such as the existence of hyperammonia-producing bacteria (HAB) and how they can be used to improve N efficiency in ruminants (Krause et al., 2014). The excess of proteolysis by bacteria in the rumen can be detrimental (Bento et al., 2015), isolation and characterization of HAB in animals fed tropical diets or supplemented with rumen-degradable proteins. This work investigated the bacterial community diversity of the rumen of Nellore steers fed tropical forages, with or without casein supplementation (Table 1). Most bacteria produced a variety of VFA from trypticase fermentation, with a predominance of acetic acid, propionic acid, butyric acid, isobutyric acid, isovaleric acid and formic acid. All the hyper-ammonia-producing bacteria were Gram-positive, by ranking VFA as high, medium and low production for concentration of fermentation end-products.

The proteolysis in the rumen can be beneficial to the animal host if the products are transformed into digestible microbial protein. The PEP are intermediates in the

ruminal bacteria conversion of protein ingested ammonia and are a point at which the rate of breakage can be controlled. The rate of protein digestion in the rumen often exceeds the capacity of microorganisms to incorporate the AA released. This imbalance results in deamination and loss of ammonia through the rumen wall, one of the main causes of inefficient N retention by ruminants (Falconer and Wallace, 1998; Krause et al., 2014).

The predominant proteases in ruminal contents are modified by diet and also vary greatly between animals, these variations are due mainly to the amount of soluble protein in diet. The proteolysis is an important function of ruminal microorganisms because it provides PEP and subsequently, AA for growth and energy production. Mixed cultures of bacteria use PEP faster than AA and ammonia is captured by passive diffusion (Russell and Strobel, 2005).

Microbial protein synthesized in the rumen represents 40 to 90% of the protein that reaches the small intestine, although more than 50% of this microbial protein synthesis can be degraded to ammonia in the rumen. Microbial N recycling in the rumen occurs as a result of both break and degradation of protozoa and bacteria. According to Lapierre and Lobley (2001), around 45 to 60% of microbial N can be derived from ruminal ammonia. But, they believe that this N is recycled several times, increasing the chance of converting 20 to 50%. This explains how the production of N from the hepatic urea in some circumstances may be more important than dietary N.

The pool of PEP, AA and ammonia in the rumen tends to increase after 2 to 4 h pots ingestion of the feedstuff. Certainly, the concentration of PEP can range from 10 to 150 mg N/l, and that of AA from 0.1 to 16 mg N/l. However and according to Nolan and Dobos (2005), this variation depends on the protein degradability and conditions for microbial growth (Krause et al., 2014).

Recycling within the rumen microbial matter is extensive and affects the feedstuff conversion efficiency, normally because energy is required for re-synthesis of microbial protein and microbial protein is degraded submitted to deamination. Maximizing the microbial protein synthesis and pass their flow for duodenum (Argyle and Baldwin, 1989; Fatehi et al., 2015).

EFFECT OF PROTEIN SOURCES ON MILK PRODUCTION AND COMPOSITION

The use of supplemental with AA protected rumen degradation has been useful in lactation cows feeding high production to meet the needs of protein and providing satisfactory results in the production of milk, especially during the first weeks of lactation. Infusions of casein in the abomasum or intestine, increases the milk production (Chalupa and Sniffen, 1991; Larsen et al., 2015; Hills et al., 2015). However, in Brazil according to Santos and Greco (2007) from 127 compared 88 experiments with cows in milk production, where the soybean meal was replaced partly or wholly by RUP rich sources, have concluded that only 17% of cows showed an increase in milk production due to the processing of soybean meal. The possible explanation of the absence of positive effect can be related with the reduction of rumen microbial protein synthesis by lack of RDP; or low quality of the RUP source in terms of essential AA balance or still low digestibility of RUP sources in the small intestine.

CONCLUSIONS

The efficient nitrogen metabolism in ruminants depends on complex interaction energy and various nutrients in the gastrointestinal tract and tissues. Nitrogen is not used efficiently by ruminal microorganism, unless concurrency in the diet with supplements rich in energy. Bacteria, fungi and protozoa, are important in the digestion of fiber in feedstuff. However, stimulating the growth of microorganisms in the rumen increased and high quality protein can be absorbed in the small intestine of the animal, resulting in better production. However, it is not necessary to provide excessive protein in the diet because it is lost through urine, which economically is not desirable, for efficient production systems.

Conflict of interests

The authors have not declared any conflict of interests.

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Abbreviations

AA, Amino acids; **ATP,** adenosine triphosphate; **CNE,** non-structural carbohydrates; **CP,** crude protein; **CH_4,** methane; **DM,** dry matter; **GDH,** glutamate dehydrogenase; **GS,** glutamine synthetase; **HAB,** hyper-ammonia-producing bacteria; **N,** nitrogen; **NPN,** non-protein nitrogen; **PEP,** peptides; **RDP,** protein degraded in the rumen; **RUP,** protein is not degraded in the rumen; **VFA,** volatile fatty acid.

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Full Length Research Paper

Helicobacter pylori vacA genotyping in relation to *cagA* status, ultra-structure of gastric mucosa and clinical outcomes in Egyptian patients

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***Helicobactor pylori (H. pylori)* has been strongly associated with gastritis, peptic ulcer and is linked to an increased risk of gastric cancer. The cytotoxin-associated gene product (*cagA*) and the vacuolating cytotoxin (*vacA*) have been implicated as two major virulence factors of *H. pylori.* Since there is an increasing evidence that genetic variability of *H. pylori* may have clinical importance, we aimed to evaluate different *vacA* genotypes and reveal its relationship with endoscopic and transmission electron microscopy (TEM) findings among *H. pylori* infected Egyptian patients. Forty *H. pylori* infected patients possessing *vacA* gene who underwent upper endoscopic examination were considered to be infected with *H. pylori* when rapid urease test and detection of 16S rRNA in gastric biopsy recorded positive. Both *vacA* and *cagA* genotypes were detected by polymerase chain reaction (PCR). The TEM was performed to assess the ultra-structure of the gastric mucosa. Four *vacA* genotypes were identified, the most prominent was the *s2/m2* allele combination (52.5%) followed by *s1/m1* (27.5%), *s1/m2* (17.5%) and *s2/m1* genotype was found just in one *H. pylori* strain (2.5%). There were significant correlations between *vacA s2/m2* and gastritis (65.2%), and *vacA s1/m1* and peptic ulceration (57%). The *cagA* gene was associated with 38% of *vacA* genotypes and 60% of which were significantly associated with *vacA s1/m1* genotype with the development of severe gastritis reaching up to gastric ulcer. The TEM revealed *H. pylori* spiral and coccoid forms, cytoplasmic vacuolar degeneration caused by *vacA*, swollen mitochondria and dilated rough endoplasmic reticulum. In Egypt where prevalence of *H. pylori* infection is high, genotyping of *H. pylori* virulence factors can help to predict patients who are at a high risk of related gastroduodenal diseases. Although *H. pylori* with *vacA s2/m2* genotype is mostly related to low level of virulent strains yet, significant crosstalk between *H. pylori* strains harboring both *vacA s1/m1* and *cagA* gene provides crucial insights into virulence of high level.**

Key words: *Helicobactor pylori*, *vacA* genotyping, *cagA*, gastritis, peptic ulcer.

INTRODUCTION

Helicobacter pylori is a spiral, Gram-negative bacterium that inhabits the stomachs of approximately half of the world's population (Warren and Marshall, 1983). Infections with *H. pylori* may induce gastritis, gastric and

duodenal ulcers and even is linked to an increased risk of gastric cancer (Khalifa et al., 2010). *H. pylori* secrete many of the proteinaceous factors that are important for initial colonization and subsequent persistence in the stomach. Two major virulence factors of *H. pylori* have been described; the cytotoxin-associated gene product (*cagA*) and the vacuolating cytotoxin (*vacA*). Both play a crucial role in determining the clinical outcome of *H. pylori* infection and their genes could serve as epidemiological markers (Marie, 2012).

The VacA toxin binds to target cells and is internalized causing severe "vacuolation" that has been attributed to the formation of VacA anion selective channels in membranes. In addition to the induction of vacuolation, VacA exerts a variety of other effects on target cells, including disruption of mitochondrial functions, stimulation of apoptosis and blockade of T-cell proliferation (Palframan et al., 2012).

The *vacA* gene, encoding the vacuolating toxins is virtually present in all strains of *H. pylori*. Polymorphism in *vacA* gene sequence has been identified in three variable regions; signal (s) region, mid (m) region and intermediate (i) region. Two types of allelic variations in the s-region and m-region are classified as s1 or s2 and m1 or m2 respectively. Therefore, the *vacA* gene of a given strain is composed of any of the possible combination of s and m region sequence type (Atherton et al., 1995). Type *s1/m1 vacA* causes more epithelial cell damage than type *s1/m2,* whereas type *s2/m2* and the rare *s2/m1* are non-toxic (Letley et al., 2003; Argent et al., 2008). In our study we aimed to evaluate different *vacA* genotypes and to reveal its relationship with endoscopic and TEM findings among *H. pylori* infected Egyptian patients.

MATERIALS AND METHODS

Patients and specimens

Forty *H. pylori* infected patients possessing *vacA* genes who underwent upper endoscopy for various dyspeptic symptoms at Endoscopy Unit, Theodor Bilharz Research Institute (TBRI) Hospital in-between March 2012 to April 2013 were enrolled in this study. The mean age of the patients was 50.05 years (range, 17-76 years), 29 were males and 11 were female. None of the patients had received non-steroidal anti-inflammatory drugs, as well as antibiotics, H2 receptors antagonists or proton pump inhibitors in the past four weeks prior to the study. Thorough endoscopic examination of the oesophagus, stomach and duodenum and clinical condition of the patient (gastritis, peptic ulceration, normal endoscopy and other findings) were assessed. Four antral biopsy specimens were obtained from each patient. This study was approved by TBRI Institutional Review Board (IRP) FWA 00010609 and informed consent was obtained from each subject before endoscopic examination. A patient was considered to be infected with *H. pylori* when rapid urease test and detection of 16S rRNA in gastric biopsy specimen were recorded positive.

Rapid urease test for *H. pylori* infection

One gastric biopsy was inserted and completely immersed in the reagent solution which is composed of urea, phenol red and stabilizers (Bussero, Milan, Italy). The positive sample turns color from yellow to magenta within 30 min.

DNA extraction

Two antral gastric biopsy specimens for each patient were stored in 0.9% saline at –70°C until used for polymerase chain reaction (PCR) assay. DNA for PCR was extracted directly from antral gastric biopsy specimens using the QIAamp tissue kit provided by (Wizard SV Genomic DNA Purification System, USA) according to the manufacturer's instruction. The DNA extracts were stored at –20°C until used for PCR assays.

PCR-based *H. pylori* detection, *vacA* genotyping and *cagA* status

PCR assays were performed in a volume of 50 µl with approximately 10 µg of total extracted DNA. PCR amplifications were carried out in Gene Amp PCR system 9700 (Bio Rad T100 thermal cycler). The 50µl reaction mixture consisted of 10 ug of the extracted DNA, x1 PCR buffer, 1.5 mM Magnesium Chloride, 200 µM of each dNTP, 20 pmol of each primer (Table 1) and 1U Taq DNA polymerase (Promega).

PCR amplification of the *H. pylori* 16S rRNA

Amplification was carried using the following cycling parameters: An initial denaturation at 95°C for 5 min and 35 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. This was followed by a final extension of 72°C for 10 min (Chisholm et al., 2001).

PCR amplification of the *H. pylori vacA* genotypes

The following cycling parameters were used: an initial denaturation at 95°C for 4 min and 35 cycles of 95°C for 1 min, 52°C for 1 min and 72°C for 1 min. This was followed by a final extension of 72°C for 10 min (Falsafi et al., 2009).

PCR amplification of the *H. pylori cagA* gene

The following cycling parameters were applied: an initial denaturation at 94°C for 5 min and 35 cycles of 94°C for 1 min, 59°C for 1 min and 72°C for 1 min. This was followed by a final

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Table 1. Primer sequences and size of expected amplicon of each PCR assay.

Target genes	Primers sequences (5'- 3')	Size of Amplicon (bp)	References
16S rRNA	F: CTG GAG AGA CTA AGC CCT CC R: ATT ACT GAC GCT GAT TGT GC	110	Chisholm et al., 2001
cagA	F:AATACACCAACGCCTCCA R:TTGTTGCCGCTTTTGCTCTC	400	Falsafi et al., 2009
vacA:			
vac A (s)	F: ATGGAAATACAACAAACACAC R: CTGCTTGAATGCGCCAAAC	*s1*:259 *s2*:286	Falsafi et al., 2009
vac A (m)	F:CAATCTGTCCAATCAAGCGAG R: GCGTCTAAATAATTCCAAGG	*m1*:570 *m2*:642	

extension of 72°C for 10 min (Falsafi et al., 2009). Each PCR product was separated on a 2% Agarose gel and 50 bp ladder was used as DNA molecular weight standard. In each PCR assay, negative control (lacking DNA) was included.

Transmission electron microscopy (TEM) examination of gastric mucosa

Segments from each gastric biopsy specimens were collected from *H. pylori* infected patients. Segments were immediately fixed for 2 h in equal volumes of glutaraldehyde 4% and caccodylate 0.2 M. The fixed segments were washed in equal volumes of Sacchrose 0.4 M and caccodylate 0.2 M for 2 h and incubated in osmium tetroxide 2% and caccodylate 0.3 M for 1 h. The samples were washed with distilled water and finally dehydrated in ascending grades of ethyl alcohol for 5 min each (30, 50, 70 and 90%) then absolute alcohol 100% for 10 min for three times. Substitution in a mixture of epoxy resin and ethyl alcohol in equal volumes for 1 h was done. Impregnation in pure resins using Epon A and Epon B in equal volumes making three washes on three successive days was done. The specimens were embedded in epoxy resin to which was added an accelerator DMP 30 in special capsules then left in oven at 60°C for 2 days to polymerize and harden. The thin sections were stained with uranyl acetate and lead citrate and were examined with TEM to assess the interaction between *H. pylori* and the ultra-sructure of the gastroduodenal epithelial cells (Bai et al., 2010).

Statistical analysis

Results are expressed as number (%). Comparison between categorical data was performed using Chi square test. Statistical Package for Social Sciences (SPSS) computer program (version 19 windows) was used for data analysis. P value ≤ 0.05 was considered significant and < 0.01 was considered highly significant.

RESULTS

H. pylori infection in all patients was confirmed by rapid urease test and detection of 16S rRNA of *H. pylori* by agarose gel electrophoresis (110 bp) in gastric biopsy specimens (Figure 1).

Clinical outcomes

During upper gastrointestinal endoscopy of the studied 40 *H. pylori* infected patients possessing *vacA* genes, their gastroduodenal mucosa had developed gastritis in 23 (57.5%), whereas 7 (25%) had peptic ulceration (including one with suspected malignant ulcer). Other endoscopic findings as; oesophageal varices, gastro-oesophageal reflux, gastric prolapse, hiatus hernia were revealed in 9(22.5%) cases and only one patient had normal gastric mucosa.

vacA genotyping

The *vacA* s- and m- region genotype were determined in all the studied 40 *H. pylori* strains. The *vacA s1* allele was found in 18 (45%) of the *H. pylori* strains and the *s2* allele was found in 22 (55%) (Figure 2, Table 2). Whereas *vacA m1* allele was found in 12 (30%) of the *H. pylori* strains and the *m2* allele was found in 28 (70%) (Figure 2, Table 2). Four *vacA* genotypes were identified in the study, the most prominent one was the *s2*/*m2* allele combination (52.5%) followed by *s1*/*m1* (27.5%), *s1*/*m2* (17.5%) and *s2*/*m1* genotype was found just in one *H. pylori* strain (2.5%) (Figure 2, Table 3).

Relationship between *vacA* genotypes and clinical outcome

Upon endoscopy, 15 out of 23 *H. pylori* infected patients with gastritis (65.2%) were significantly associated with *vacA* genotype *s2/m2* allele combination (P< 0.01), whereas in peptic ulceration, 4/7 (57%) of them were significantly associated with *vacA s1/m1* genotype (P<

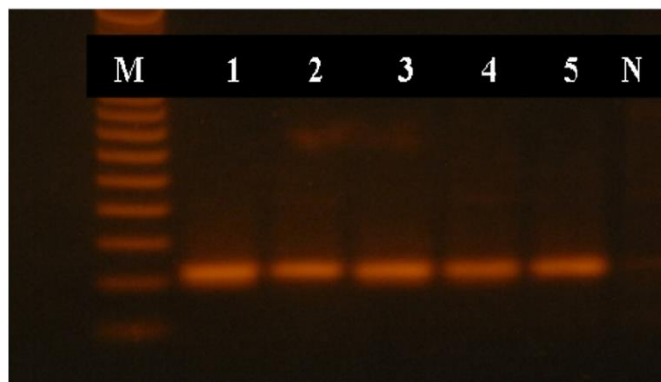

Figure 1. Agarose gel electrophoresis of PCR products of *H. pylori* 16S rRNA positive gene (110 bp) from gastric biopsy on agarose gel. Lane M: molecular weight marker (ladder 50 bp). Lane (1-5): Positive cases of *H. pylori* possessing 16S rRNA gene. Lane N: negative control.

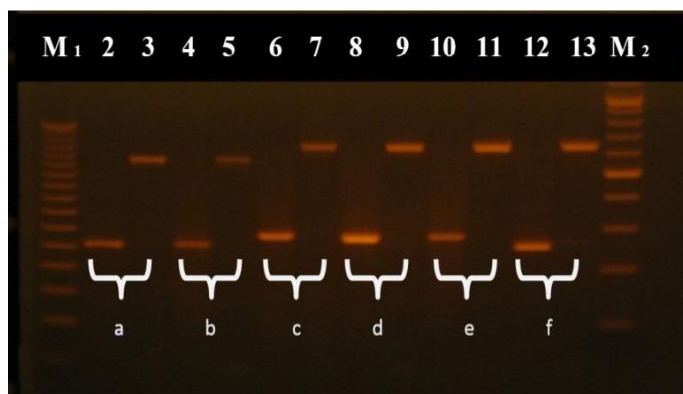

Figure 2. Agarose gel electrophoresis of PCR products of *H. pylori vacA* gene alleles from gastric biopsy, *s1*:259 bp, *s2*:286 bp, *m1*:570 bp, *m2*:642 bp. Lane M1: molecular weight marker (ladder 50 bp). Lane (2, 3) and (4, 5): Genotype of samples a and b which was *(s1/m1)*, Lane (6, 7), (8, 9) and (10, 11): Genotype of samples c, d and e which was *(s2/m2)*, Lane (12, 13): Genotype of sample f which was *(s1/m2)*. Lane M2: molecular weight marker (ladder 100 bp).

0.01). Normal gastric mucosa was found in one *H. pylori*-infected patient of *vacA s2/m1* genotype (Table 3).

Relationship of *vacA* genotypes to *cagA* status and clinical outcomes

The *cagA* gene was detected in 38% (15/40) of the studied *vacA* genotypes strains (Figure 3); 60% of which were significantly associated with *vacA s1/m1* genotype (P=0.041) with the development of severe gastritis

Table 2. Prevalence of *vacA* genotype alleles among the studied 40 *H. pylori* strains.

***vacA* Genotype Alleles(N=40)**	
Genotype alleles	**N (%)**
vacA	
s1	18 (45%)
s2	22 (55%)
vacA	
m1	12 (30%)
m2	28 (70%)

reaching up to gastric ulcer, 33.3% (5/15) of *cagA*-positive strains were associated with *vacA s1/m2* genotype and only one strain was of *s2/m2* genotype.

Analysis of TEM examination

Electron microscopy revealed *H. pylori* in its spiral and coccoid forms (Figure 4), as well as cytoplasmic vacuolar degeneration played by the vacuolating toxin (Figure 5). Ultrastructural examination of the tiny gastric biopsies revealed tissue debris of exfoliating degenerated mucosal epithelium with exposure of blood vessels of the lamina propria in many examined ultrathin sections with large lipid accumulation. Inflammatory lymphocytic cells was evident in the examined sections. Curved *H. pylori* and cluster of unidentified bacilli nearby degenerated mucosal gastric structures were depicted in *H. pylori* positive cases. Also, gastric mucous cells with their characteristic granules resting on intact lamina propria were the main cells seen in most of the examined sections. Many of them showed vesiculated endoplasmic reticulum and vacuolated cytoplasm.

DISCUSSION

Little is known about the geographic distribution of *H. pylori* genotypes in distinct regions, particularly in Egypt. The *vacA* genotypes and its relationship to clinical outcomes, *cagA* status and TEM findings were investigated in *H. pylori* strains from 40 infected Egyptian patients possessing *vacA* gene.

Different results have been reported in studies related to *vacA* s and *H. pylori* strains. In the current study, the *vacA s2* allele was the most predominant genotype (55%) followed by *vacA s1* (45%). Our findings are similar to an earlier report from Egypt (Van Doon et al., 1999), Korea (Kim et al., 2001) and Turkey (Ozbey and Aygun, 2012). Al Quabandi et al. (2005) reported that North Africans

Table 3. Relationship between *H. pylori vacA* genotypes and clinical outcomes in 40 *H. pylori* infected patients.

Endoscopic findings	*vacA* genotypes			
	***s1/m1* (n= 11)**	***s1/m2* (n= 7)**	***s2/m2* (n= 21)**	***s2/m1* (n= 1)**
Gastritis (n= 23)	5 (21.7)	3 (13.0)	15 (65.2) **	0 (0.0)
Peptic ulcer (n= 7)	4 (57.1) **	1 (14.3)	2 (28.6)	0(0.0)
Normal gastric mucosa (n= 1)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100)
Others# (n=9)	2 (22.2)	3 (33.3)	4 (44.4)	0 (0)

Data were expressed as number (%). # Other endoscopic findings which include; oesophageal varices, gastro-oesophageal reflux, gastric prolapse **P< 0.01= highly significant.

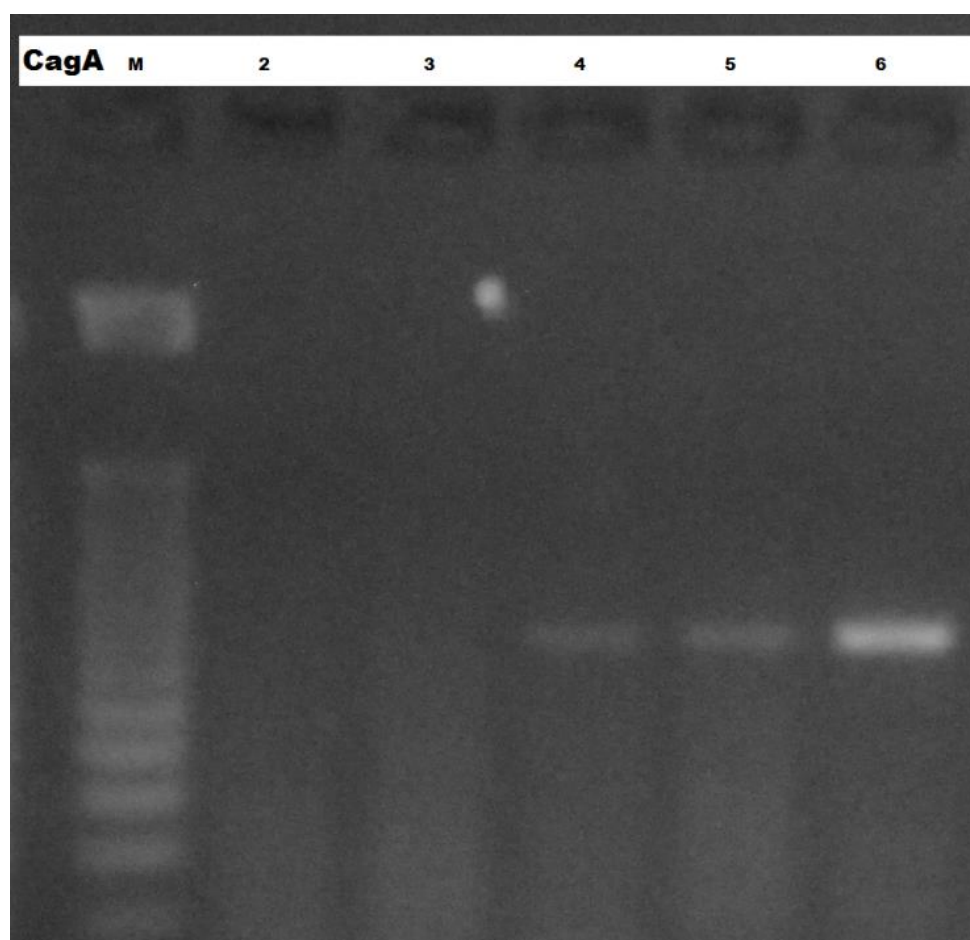

Figure 3. Agarose gel electrophoresis for PCR products of *H. pylori* of *cagA* status. Lane M: Molecular weight marker (50 bp). Lane 2: Negative control. Lanes 4-6: Positive *cagA* gene (400 bp).

were predominantly infected with the *s2* type. In contrary to our results, the previous study from Kuwait reported that *vacA* s1 and s2 types were detected in approximately equal numbers in biopsies obtained from patients of Middle Eastern origin. Previous reports from Cyprus (Krashias et al., 2013) and Saudi Arabia (Sugimoto et al., 2009) revealed that *s2* is the main allele among their *H. pylori* strains.

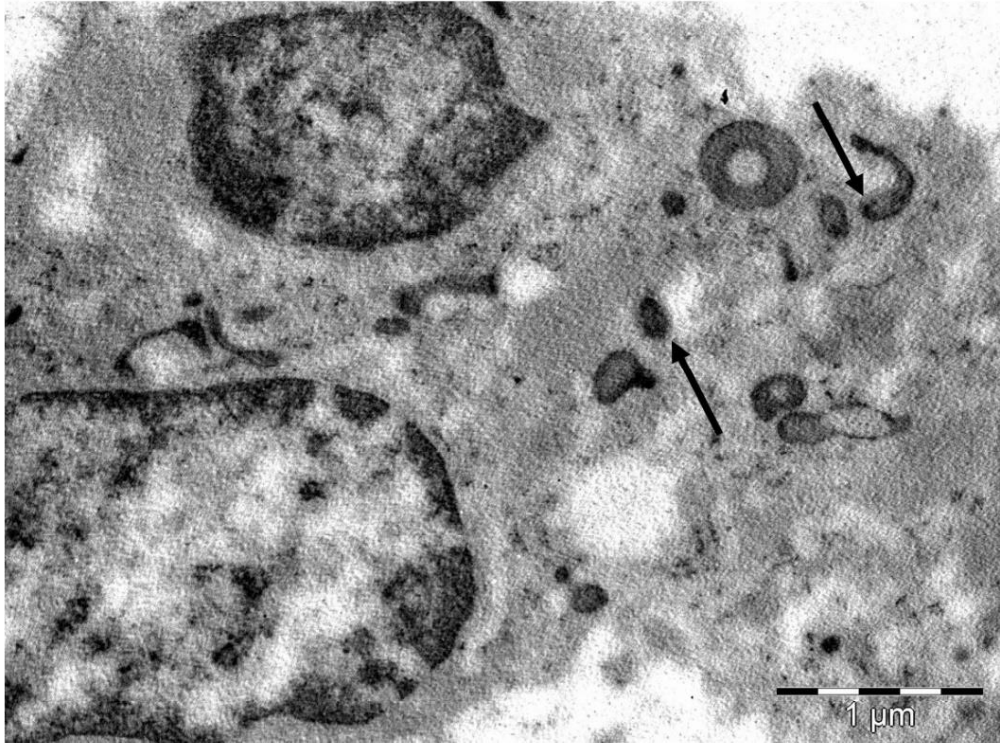

Figure 4. Electron micrograph of gastric biopsy showing spiral (arrow) and coccoid (arrow) forms of *H. pylori.*

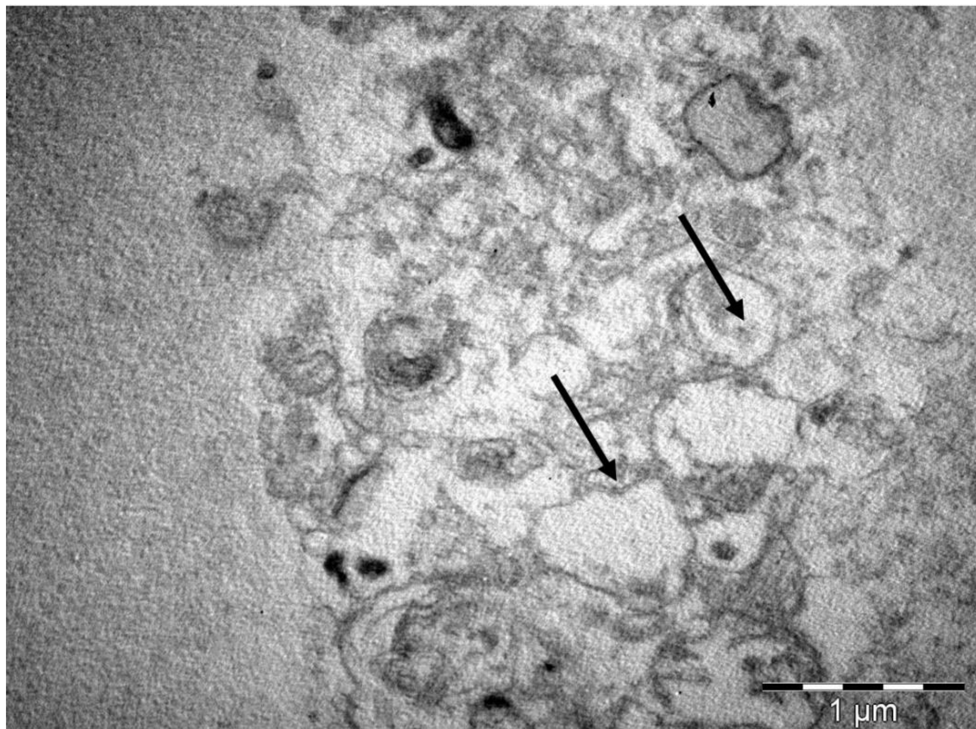

Figure 5. Electron micrograph of *H. pylori* infected gastric mucosa showing cytoplasmic vacuolar degeneration (arrow) caused by the vacuolating toxin.

The m-region encodes the *vacA* binding site to host cells, which appears to be more effective in the *m1* than in *m2* forms (Ferreira et al., 2014). Similar to previous studies in Turkey, Iraq, Iran, Saudi Arabia (Hussein, 2010) and China (Chung et al., 2010), *m2* was predominantly found in all studied *H. pylori* strains (70%) previously reported. However, this was inconsistent with previous results obtained from Egypt where equal distribution of *vacA m1* and *m2* was found (Amer et al., 2013). Genetic variation within virulence factors may account for differences in the pathogenic properties of strains, and thus may help to explain the discrepancies between the number of infected individuals and those that end up developing gastric cancer (Ferreira et al., 2014).

The allele combination between *vacA s* and *vacA m* genotypes and their association to gastroduodenal disorders differ greatly (Nimri et al., 2006). In this study, the prevalence of the *vacA* genotypes *s1m1* was 27.5%, and 57.1% of which was highly significant in relation to peptic ulcer. This is in agreement with previous studies in which association between this genotype and severe gastric outcomes was recognized (Marie, 2012). It is worth to mention that, in these cases long curved virulent spiral forms of *H. pylori* were detected at the level of electron microscopy in association with ulcerated and degenerated mucosal lining. Also, multiple bacterial microorganisms exhibiting curved appearance were seen among the mononuclear inflammatory cells undergoing apoptotic changes and in between the debris of degenerated mucosal cells. It was reported that the long spiral form facilitate the penetration and movement of the microorganism through the mucous gel. *H. pylori* lacking the spiral form loses its infectiousness (Bai et al., 2010). Moreover, in this study cytoplasmic vacuolar degeneration of mucosal cells with swollen mitochondria and dilated rough endoplasmic reticulum denoting the presence of the *vacA* gene toxic effect was an important finding. (Atherton et al., 1995) and Bai et al. (2010) reported that the pivot role in cell damage induced by *H. pylori* is played by vacuolating toxin.

The significant association between *vacA s1/m1* genotypes and gastric carcinoma development has been substantiated by meta- analysis using reports of patients from diverse geographic origin, including Western, Middle East, Latin America and Africa countries. In an observational longitudinal study from Spain, the progression of premalignant lesions was more frequent in patients infected with *vacA s1/m1* strains than those infected with less virulent *vacA s2/m2* strains (Ferreira et al., 2014). Our data emphasizes the significant association of the most predominant (52.5%) allele combination *s2/m2* with gastritis (65.2%). This predominance goes in accordance with Benenson et al. (2002) and El-Gharbawy et al. (2006). This similarity in *H. pylori* genotypes in three neighboring countries, Egypt, Jordan, and Gaza strip, indicates a geographic influence, which was reported by Abu Amra (2010). Whereas in Egypt, Amer et al. (2013), reported that all possible combination of vacA s1 with m were recognized in their work, in addition, *H. pylori* virulence could not be predicted in relation to different genotypes.

The prevalence of *cagA* (38%) in this study was in agreement to previous report from Egypt (El-Garbawy et al., 2006) and lower than recent studies from Turkey (71.4%) (Ozbey and Aygun, 2012), Korea (97%) (Kim et al., 2001), Malaysia (94%) (Ramelah et al., 2005) China (96.3%) (Zhou et al., 2004) and Kuwait (53%) (Al Quabadi et al., 2005).

Regarding the relationship between *H. pylori* genotypes and clinical outcome, we found a significant association between *cagA* status and *vacA* s1/m1 genotype with development of severe gastritis reaching up to gastric ulcer. These findings are in agreement with Marie (2012) and Ozbey and Aygun (2012).

The attribution between the presence of *cagA* and severe clinical outcome has been a controversial point; as it was previously found that colonization with cagA positive strains has been associated with a fivefold increased risk for diagnosis of duodenal or gastric cancer (Diab et al., 2009). Whereas in other studies the occurrence of gastric malignancy was independent of *cagA* status and others implicated the pivotal roles of other virulence factors (*cag E, cag T*, *vac A* and *bab A*) in the aetiology of gastric cancer (Marie, 2012).

Conclusion

In Egypt where prevalence of *H. pylori* infection is high, genotyping of *H. pylori* virulence factors can help to predict patients who are at a high risk of related gastroduodenal diseases. Although *H. pylori* with *vacA s2/m2* genotype is mostly related to low level of virulent strains yet, significant crosstalk between *H. pylori* strains harboring both *vacA s1/m1* and *cagA* gene provides crucial insights into virulence of high level.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Virulence profiles of clinical and environmental *Pseudomonas aeruginosa* isolates from Central Morocco

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The pathogenic potential of *Pseudomonas aeruginosa* comes from the expression of many secreted and cell surface virulence factors, and its biofilm formation. This study aimed to investigate and compare the virulence profiles of 123 clinical and environmental *P. aeruginosa* isolated in Meknes (Morocco). Using suitable culture media, phenotypic screening evaluated the production of β-haemolysin, caseinase, lipase, lecithinase, pyocyanin and pyoverdin, as well as the ability to swim, swarm and twitch. Biofilm formation kinetics was assessed using microtiter test plates. Data analysis was performed using Statistic Package of the Social Science software (version 21.0). High percentages of strains expressed caseinase (99.2%), β-heamolysin (95.1%), lipase (100%) and lecithinase (100%). 95.9% of isolates produced either pigment. All strains were able to swim, warm and twitch, at different levels. All strains were biofilm producers, and the evolution of adherent biomass over time varies greatly from strain to strain. Significant positive correlations were observed between proteolytic and hemolytic activities; biofilm formation and twitching; as well as swimming, swarming and twitching motilities. Twitching and swimming were significantly higher in environmental strains, which were also quickly adhered and formed denser biofilms. Clinical strains showing significantly higher proteolytic activity were isolated from cardiology ward, and those with higher twitching and denser biofilm were from the thoracic service. Inpatient strains were significantly earlier producer of denser biofilm than outpatient ones. *P. aeruginosa* strains tested have a collection of virulence markers required to cause disease in different tissues. Such bacteria present a serious therapeutic challenge for treatment of both community-acquired and nosocomial infections.

Key words: Biofilm, clinical, environmental, *Pseudomonas aeruginosa*, virulence factors.

INTRODUCTION

Pseudomonas aeruginosa (*P. aeruginosa*) is an important human opportunistic pathogen that causes serious nosocomial infections. It is associated with significant morbidity and mortality, particularly in immuno-compromised hosts and vulnerable patients (Kerr et al., 2009). In fact, *P. aeruginosa* infections are especially

difficult to treat because of its versatility (Lister et al., 2009) and intrinsic/acquired antibiotic resistance (Breidenstein et al., 2011), as well as its capacity to form a protective biofilm (Breidenstein et al., 2011) and to express many secreted and cell-associated virulence factors (Goodman et al., 2004).

P. aeruginosa is armed with a large collection of virulence factors that allow it to survive in both different hosts and the environment.These factors are involved in the various stages of the infection process thereby allowing *P. aeruginosa* to colonize the host (Lau et al., 2005; Lyczak et al., 2000). Membrane factors are involved in the adhesion and motility of *P. aeruginosa* and allow the host colonization. They include flagellum, type IV pili, lipopolysaccharide, a type III secretion system, and alginate (Pier, 2002).Throughout its growth, this bacterium releases several metabolites including mainly, exotoxins, exoproteases, heamolysins and chromophores. These factors cause extensive tissue damage, as well as facilitate bacterial multiplication and the spread in the host tissues (Van Delden and Iglewski, 1998). Biofilm production is also known as an important determinant of pathogenicity in *P. aeruginosa* infections, it has been recognized as the principal mechanism associated with prolonged and recurrent infections (Wareham and Curtis, 2007).

The purpose of the present study was to investigate and compare the production, *in vitro*, of some enzymes (β-haemolysin, caseinase, lipase and lecithinase) and pigments (pyocyanin and pyoverdin) involved in the virulence of 123 *P. aeruginosa* isolates obtained from environment and clinical samples. Their ability to swim, swarm and twitch, as well as their biofilm formation kinetics were also examined. Possible correlations between these parameters were equally sought.

MATERIALS AND METHODS

Bacterial strains

A total of one hundred and twenty three environmental and clinical isolates of *P. aeruginosa* from Meknes city (Morocco), which had been identified and tested for antimicrobial susceptibility in a previous study (Maroui et al., 2016), were included in this work. These strains were collected from June 2012 to June 2014.

Environmental strains (n = 55) were from soil (n = 20), aliments (n = 3), amurca olive (n = 6), rivers water (n = 16), wells (n = 5) and public swimming pools (n = 5). Clinical strains (n=68) were isolated from male (n = 51) and female (n = 17), the distribution of these isolates depending on the levy type was as follows: distal bronchial levy protected (n = 6), pus (n = 35), urine (n = 18), pleural fluid (n = 4), biopsy (n = 2), blood cultures (n = 1), bronchial aspirate (n = 1) and vaginal levy (n = 1). A third of these isolates were from outpatients and two thirds from inpatients of various hospital wards mainly intensive care (12/45) and burn ward (12/45).

Screening for some virulence factors

Few colonies grown overnight on nutrient agar at 37°C were suspended in 4 ml of Luria Bertani (LB) broth. The density of this suspension was adjusted to 0.5 of the McFarland standard. 3 or 10 µl of this suspension were added to several media containing the appropriate substrates as described below. All experiments were performed in triplicate. Pigments production was assessed qualitatively by observing the dyes excreted into the culture medium surrounding the colonies.

Hemolytic activity

The strains were tested for β-hemolytic activity on blood agar plates prepared with Columbia agar supplemented with 5 % human blood. Ten microliters of each bacterial suspension was placed in 5-mm-diameter well cut into the agar and incubated at 35°C for 24 h. The presence of a clear colourless zone surrounding the well indicated β-hemolytic activity (Gerhardt et al., 1981).

Proteolytic activity

Casein hydrolysis was tested on Mueller Hinton agar containing 10% (w/v) skimmed milk (Gudmundsdóttir, 1996).Ten microliters of each bacterial suspension was placed in 5 mm-diameter well cut into the agar and incubated at 35°C for 24 h. The presence of a clearing zone around the well indicated proteolysis of casein, and the diameter of the clearing zone reflects the intensity of the exoenzyme released. Reference strain *P. aeruginosa* ATCC 27853 was used as a control in this test.

Lecithinase and lipase activities

Lipase and lecithinase activities were tested on egg yolk agar plate prepared with phosphate buffered saline, containing egg yolk to 5 and 1.5% bacto-agar. Three microliters of each bacterial suspension was placed onto the agar and incubated at 35°C for up to five days. The appearance of an opaque precipitate that spreads beyond the edge of the colony indicated lecithinase activity and the formation of an iridescent sheen that can be seen when the plate is held at an angle to a light source indicated lipase production (Forbes et al., 2007; Koneman et al., 2006). Reference strain *P. aeruginosa* ATCC 27853 was used as a control in this test.

Pyocyanin and pyoverdin production

The strains were streaked on Cetrimide agar and king B agar, and incubated for 24 h at 37°C for visual analysis of pigment production. Blue pigment was interpreted as pyocyanin, yellow-green and fluorescent indicated the pyoverdin production, and red-brown pigment was recorded as pyorubin.

Motility assays

In the following 3 tests, 90 mm plates were filled with 30 ml of medium, dried before use, and inoculated in triplicate experiments. The plates were incubated at 30°C for 24 h. Swimming and

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swarming motilities were assessed qualitatively by measuring the turbid zone formed by the bacterial cells migrating around the point of inoculation. For twitching, the zone of motility at the agar/Petri dish interface was visualized by gently removing the agar, the Petri dish was air dried and cells were stained with crystal violet (1%). Then the Petri dish was rinsed with distilled water, and the crystal violet-stained twitching pattern was measured. Strains were divided into four groups: no motile (no growth spreading), less motile (< 1 cm), moderately motile (1 cm ≤ and < 2 cm) and highly motile (≥ 2 cm).

Swimming

Swimming assays were done on 0.3% LB agar plates (Murray and Kazmierczak, 2006). Swim plates were inoculated by sterile toothpick with single colonies picked from a fresh nutrient agar plate.

Swarming

Swarm media were composed of 0.8% nutrient broth, 0.5% glucose and 0.5% bacto-agar (Rashid and Kornberg, 2000). Fresh isolated colonies were point-inoculated with sterile toothpick on the surface of the agar.

Twitching

Twitch plates were composed of 1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 1% bacto-agar. Bacterial colonies were inoculated with a sharp sterile toothpick inserted through the culture medium to the bottom of the Petri dish (Rashid and Kornberg, 2000).

Quantification and kinetics of biofilm formation

Biofilm formation assays were carried out in microtiter plates of polystyrene according to the crystal violet method as described by Stepanovic et al. (2007). Briefly, 160 µl of sterile LB broth was placed in each well of a sterile 96-well microtiter plate, then 20 µl of each bacterial suspension from an overnight culture of stirring (150 rpm) in LB broth was added to each well. Microplates were covered and incubated without agitation at 37°C for 2 to 24 h. All the samples were prepared in duplicate for each sampling time with a standard strain (*P. aeruginosa* ATCC 27853) and two negative controls containing no strains by plate. After incubation at regular time intervals, the non-adherent bacteria were removed by three successive washes with distilled water then the duplicate plates were air dried. Biofilm formation was visualized by staining with 1% crystal violet (200 µl per well) for 15 min at room temperature followed by rinsing thrice with distilled water, and then the plates were air dried. Biomass attached cells was quantified by solubilizing the dye in 200 µl per well of 95% ethanol.

The amount of biofilm formed was evaluated by reading the absorbance (optical density) of each well using a spectrophotometer (BIO-RAD Laboratories PR 2100), at a wavelength of 490 nm. Based on the optical density (ODi) of the samples and the average of optical density of the negative control (ODc), the samples were classified as strong biofilm producers (4xODc < ODi), moderate biofilm producers (2xODc < ODi ≤ 4xODc), weak biofilm producers (ODc < ODi ≤ 2xODc) or non-biofilm producer (ODi < ODc) (Stepanovic et al., 2007).

Depending on the incubation time required to achieve maximum adhesion, the strains were classified as early producer (t ≤ 4 h), 1/2 early producer (6 h ≤ t ≤ 10 h), 1/2 Tardy (12 h ≤ t ≤ 18 h), and tardy producer (t ≥ 20 h).

Statistical analysis

The results were analyzed using the t-test, factor analysis and one-way analysis of variance (ANOVA). Data analysis was performed using Statistic Package of the Social Science software (SPSS version 21.0 software, IBM, Chicago, USA). The *p*-values of < 0.05 were considered statistically significant.

RESULTS

Secreted virulence factors

Studied *P. aeruginosa* strains synthesized many virulence factors. Table 1 shows the repartition of tested extracellular enzymes among strains from both origins. Caseinase, β-haemolysin, lipase and lecithinase were produced by almost all strains, although with different activity levels. All environmental strains produced the four enzymes, with an exception of a strain isolated from amurca olive which did not produce the β-haemolysin. Lipase and lecithinase were also synthesized by all clinical strains, and it was noted among these that only one isolate from urine of outpatient did not produce caseinase but produced β haemolysin, and five strains isolated from different wards did not produce β haemolysin but produced low levels of caseinase. The vast majority of isolates exhibited better caseinase, β haemolysin, lipase and lecithinase than *P. aeruginosa* ATCC 27853.

Results of pigment production by *P. aeruginosa* isolates are presented in Table 2. The pyocyanin, pyoverdin and pyorubin pigments were synthesized respectively by 73.2, 65 and 2.4% of isolates studied, however 3.2% of these were non-pigmented.

Cell surface virulence markers

All strains were motile, but showed variable degrees of motility. For each, type of motility, the rates recorded for the motility phenotypes are shown in Figure 1.

Quantification and kinetics of biofilm formation

All strains were able to form biofilm on the polystyrene substrate. Their distribution according to the amount of formed biofilm for each incubation time and each origin is shown in Figure 2. 70.9% of environmental strains and 50% of clinical ones were able to form biofilms denser than that of *P. aeruginosa* strain ATCC 27853, which was found to be moderate. Results of strains classification according to incubation time required to achieve maximum adherence and quantification of that adherence are presented in Figure 3.

Table 1. Number (%) of positive *P. aeruginosa* strains for tested extracellular enzymes.

Enzyme	No. of positive strains (%)	
	Environmental strains (n=55)	Clinical strains (n=68)
β Haemolysin	54 (98.2%)	63 (92.6%)
Protease (Caseinase)	55 (100%)	67 (98.53%)
Lipase	55 (100%)	68 (100%)
Lecithinase	55 (100%)	68 (100%)

Table 2. Number (%) of positive *P. aeruginosa* strains for pigment production.

Pigment	No. of positive strains (%)	
	Environmental strains (n=55)	Clinical strains (n=68)
Pyocyanin	21(38.2%)	17 (25%)
Pyoverdin	7(12.7%)	19 (27.9%)
Pyorubin	0	1 (1.5%)
Both pyocyanin and pyoverdin	27 (49.1%)	25 (36.8%)
Both pyoverdin and pyorubin	0	2 (2.9%)
No pigment	0	4 (5.9%)

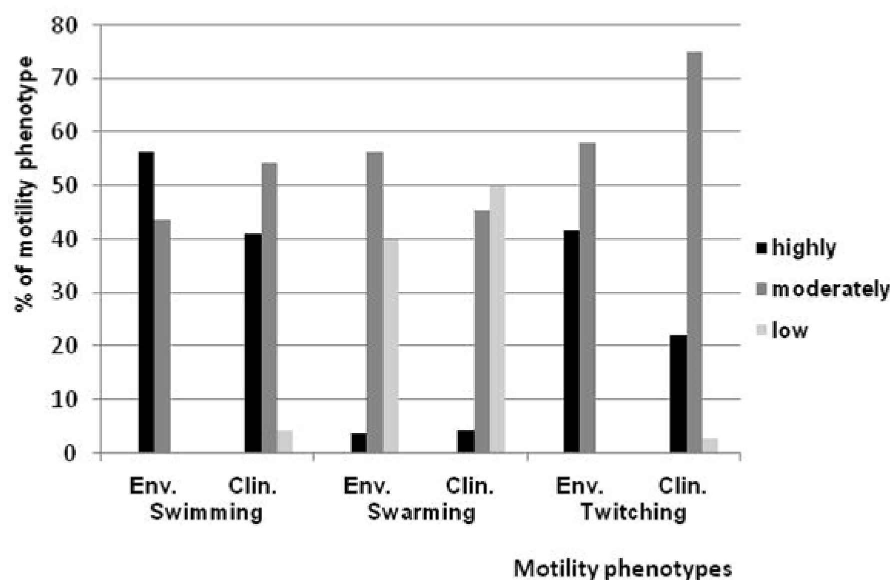

Figure 1. Motility phenotypes of *Pseudomonas aeruginosa* strains studied: highly motile (≥ 2 cm), moderately motile (1 ≤ and < 2) and low motile (< 1 cm). Env., Environmental; Clin., Clinical.

Data analysis

Comparing quantitative virulence factors studied according to the strains origin indicated that the difference observed among environmental and clinical strains was not statistically significant for caseinase, β haemolysin and swarming.

Considering all *P. aeruginosa* strains, irrespective of their origin and the isolation site, analysis of possible correlations between these virulence factors showed very significant positive correlations between β-haemolysin and protease, and also between swimming and swarming. Significant positive correlation was found between biofilm formation and twitching and between twitching and other two types of motility as well. A relatively weak positive correlation was found between

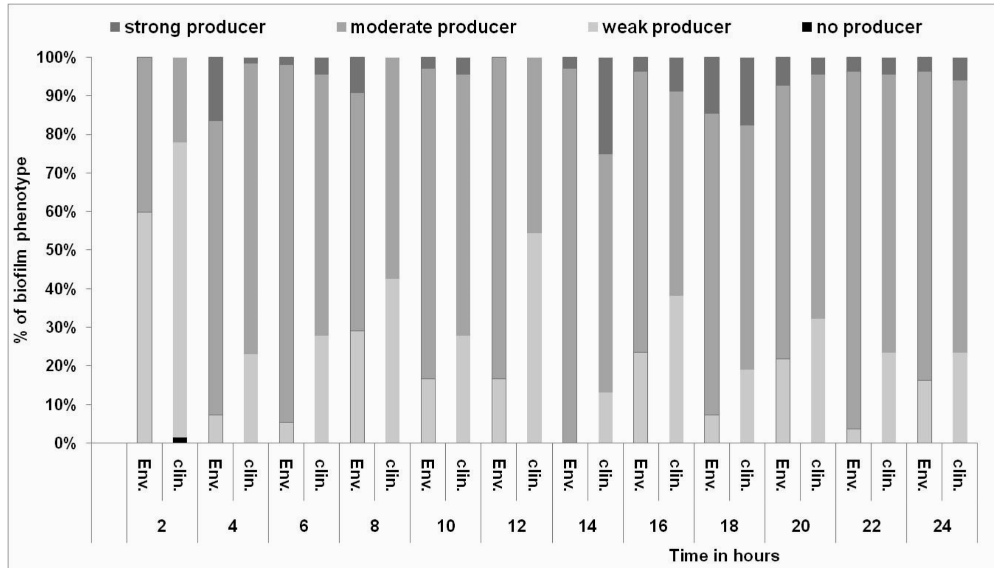

Figure 2. Comparison of the amount of biofilm formed by environmental and clinical isolates for 12 incubation times. Strong biofilm producers (4xODc <ODi), moderate biofilm producers (2xODc <ODi ≤ 4xODc), weak biofilm producers (ODc<ODi ≤ 2xODc) or non-biofilm producer (ODi<ODc). ODi: optical density of the samples, ODc the average of the negative control optical density.

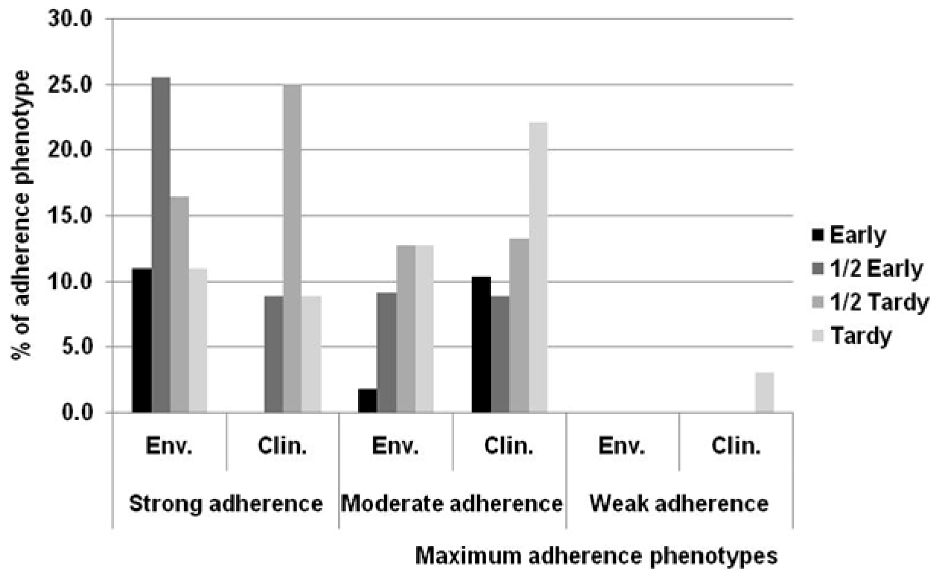

Figure 3. Adherence phenotype rates according to the incubation time required to achieve maximum adhesion. Early producer (t ≤ 4h), 1/2 early producer (6h ≤ t ≤ 10h), 1/2 tardy (12h ≤ t ≤ 18h), and tardy producer (t ≥20h). Env., Environmental; Clin., Clinical.

formed biofilm density and swimming, and swarming. Statistical significance of the effect of each categorical variable on the dependent variables studied is shown in Table 3.

The analysis of variance confirms the significant impact of the strain origin on swimming, twitching and biofilm density formed. Indeed, environmental strains had more capacity for swimming and twitching and produced denser biofilms. Significant positive impact of pyocyanin on proteolytic activity and swimming was also detected.

Significant impact of the isolation site on twitching was found. In fact, the highest twitching was observed in environmental strains especially those isolated from amurca olive. Considering clinical *P. aeruginosa* strains, patient's gender had no significant impact on virulence markers studied, but significant effects of the isolation

Table 3. ANOVA test results.

Categorical variable	*p*-values					
	Hemolytic activity	Proteolytic activity	Swimming	Swarming	Twitching	Maximum adherence
Origin	0.172	0.706	0.010	0.066	0.01	0.01
Sample	0.178	0.790	0.05	0.246	0.02	0.233
Pyocyanin	0.339	0.018	0.024	0.588	0.850	0.927
Pyoverdin	0.052	0.538	0.070	0.051	0.469	0.141
Ward*	0.343	0.01	0.073	0.084	0.017	0.013
Gender*	0.513	0.971	0.793	0.892	0.518	0.493

*For clinical strains.

ward on proteolytic activity, twitching and biofilm density were detected. Indeed, the strains that showed higher proteolytic activity were isolated from cardiology ward, and those with higher twitching motility and denser biofilm were isolated from the thoracic service.

The evolution of adherent biomass over time varies greatly from one strain to another. Comparison of biofilm formation by clinical and environmental strains showed significant differences for the incubation times of 2, 4, 6, 8, 10 and 12 h, while for other incubation times, the differences were not significant. In fact, environmental strains were quickly adhered and formed denser biofilm within a short time.

Considering environmental *P. aeruginosa* strains, comparison of biofilm formation taking into account the isolation site showed that statistically significant differences were detected only when amurca olive and soil, amurca olive and water of wells, and amurca olive and water of swimming pools were compared. Indeed, strains isolated from amurca olive formed the densest biofilms.

Taking into consideration the clinical strains, comparison of biofilm formation depending on the anatomical isolation sample showed non-significant differences. But the density of formed biofilm was significantly higher in strains isolated from the thoracic and surgery services. When inpatient and outpatient strains were compared, the differences were significant for the incubation time 2, 4 and 6 h. Therefore, inpatient strains were earlier producer of denser biofilm.

DISCUSSION

Bacterial infectivity results from a disturbance in the balance between bacterial virulence and host defense. Virulence factors, are an important determinant of pathogenicity, they allow bacteria to invade host tissues, cause disease and escape host defense mechanisms. It has been demonstrated that *P. aeruginosa* species utilizes the same virulence determinants to infect different hosts, from plants to humans (Fajardo et al., 2008).

Taking all strains included in this work, 95.1% of them exhibited extracellular β-hemolytic activity towards human erythrocytes, 99.2% produced protease (caseinase) and 100% exhibited production of lipase and lecithinase, and overall there was no significant difference among environmental and clinical strains.

These findings suggest that the tested *P. aeruginosa* are invasive isolates, as these enzymes are of known pathogenic potential. Bacterial proteases play an important role in invasiveness, host tissue damage, and evading host-defense mechanisms (Travis et al., 1995). Haemolysins involved in invasion, are considered to be a significant virulence factor of *P. aeruginosa* as indicated previously (Majtán et al., 1991; Woods et al., 1986). The most important role of bacterial extracellular lipases may be the digestion of host cellular lipids for nutrient acquisition, which results in adhering to host tissue and neighboring cells (Stehr et al., 2003). Lecithinase enzymes modulate the host immune system (Cheng et al., 1995) and play roles in cell-to-cell spread (Vazquez-Boland et al., 1992).

Among clinical isolates, 92.6% were β haemolysin producers, this result is strongly higher if compared with that reported by Stehling et al. (2008) and Holban et al. (2013) indicating percentages of 51.7 and 36.5% respectively. For lipase and lecithinase production, our results are higher than those reported by Holban et al. (2013) which signaled respectively rates of 55.8 and 88.5%. However, for caseinase, our result remains slightly lower than that found by Holban et al. (2013) indicating a percentage of 100%.

Almost all studied strains (95.9%) produced pyocyanin and/or pyoverdin that are known to have a crucial role in *P. aeruginosa* virulence. Pyocyanin, a representative pigment produced by *P. aeruginosa*, targets multiple cellular functions and contributes to acute as well as chronic infections, as it has several effects such as stimulating IL-8 release (Look et al., 2005), depressing host-response and inducing apoptosis in neutrophils (Allen et al., 2005). Pyoverdin is also a virulence marker in this pathogen, it was found that pyoverdin regulates its own secretion and the secretion of other *P. aeruginosa* virulence factors, such as exotoxin A (Lamont et al., 2002).

Among clinical isolates, 92.6% produced pyocyanin and/or pyoverdin, 61.8% produced pyocyanin, and 67.6% produced pyoverdin; these results partially agree with those reported by Finlayson and Brown (2011) which signaled respectively rates of 82.5, 57.9 and 78.5%. Other studies report the production of pyocyanin in *P. aeruginosa* isolates ranging between 41.3 and 81.5% (Fothergill et al., 2007; Iwalokun et al., 2006).

Another group of virulence factors are attachment and motility organelles, including polar flagellum and type IV pili. These cell surface structures of *P. aeruginosa* are responsible for the three most known types of motility: twitching pilus-dependent movement on solid surfaces, flagellum-mediated swimming in aqueous environments, and swarming, requiring both flagella and pili, on semisolid surfaces (Köhler et al., 2000; Rashid and Kornberg, 2000; Wall and Kaiser, 1999). In this study, it was reported that all examined strains express these motility structures. It has been demonstrated that both of these cell structures bind specifically to the host cell glycosphingolipids (Gupta et al., 1994), and that this binding event is fundamental for epithelial cell invasion and cytotoxicity (Comolli et al., 1999).

The biofilm is relevant to *in vivo* growth; it contributes to increasing infections *in vivo*. Biofilm forms a protective barrier that provides increased tolerance to antibacterials and phagocytic cells (Flemming and Wingender, 2010). In the present study, 100% of the isolates were able to form biofilm on polystyrene substrate with diversity in biofilm biomass, variable times required to achieve maximum adherence, and wide variation in the adherent biomass evolution over time. Considering the maximum adherent biomass reached for each strain, it was noted that 64 isolates form strong biofilms, 57 form moderate biofilms and 2 form weak biofilms.

In the present study, significant correlation was found between the three types of motility (mainly twitching) and biofilm density. Indeed, the flagellum and type IV pili are recognized as central components in the biofilm process. The flagellum provides mobility needed to actively approach a surface, and the type IV pili intervene in surface attachment and motility. It has been reported that type IV pili and twitching motility are involved in biofilm architecture and are responsible for the formation of microcolonies on abiotic surfaces (Chiang and Burrows, 2003).

These findings are consistent with those reported by Wolska and Kot (2013) pointing out that 100% of clinical *P. aeruginosa* strains isolated from different anatomical sites formed biofilm, and that biofilm production is in correlation with twitching motility.

The impact of pyocyanin production on biofilm formation was not detected, suggesting a similar ability of pyocyanin producers and non-producers to produce denser biofilms.

Among all tested isolates, only two strains producing pyocyanin and pyoverdin, isolated from the urine of outpatients, showed a relatively low level of virulence. In fact, these isolates showed low hemolytic and proteolytic activities, the lowest motility and the weakest biofilm formation on polystyrene surfaces.

The increased virulence observed in environmental strains could be explained by the fact that bacteria encounter a myriad of stresses in their natural environments. These stresses elicit a variety of specific adaptive responses that protect bacteria, therefore their virulence is amplified. Based on these results, it is suggested that the production of the four exoenzymes tested, pyocyanin and pyoverdin, the flagellum and type IV pili expression, and the biofilm formation as virulence markers for pathogenicity should be considered as general virulence factors, since a large number of *P. aeruginosa* strains present this phenotype.

In conclusion, *P. aeruginosa* infections typify those of a pathogen with many potential virulence factors that allow it to colonize and infect humans, animals and plants. The phenotypic characterization performed in this work clearly revealed that the studied *P. aeruginosa* strains have a collection of virulence markers required to cause disease in different tissues. Such bacteria present a serious therapeutic challenge for treatment of both community-acquired and nosocomial infections. In this context, the virulence factors produced by *P. aeruginosa* could be possible therapeutic targets to reduce the establishment of severe infections in patients presenting serious health conditions.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Determination of *mecA* expression and other resistance mechanisms in methicillin-resistant *Staphylococcus aureus* isolated from *Oreochromis niloticus* (Nile tilapia)

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Methicillin-resistant *Staphylococcus aureus* is a major cause of infection worldwide. Production of β-lactamases and penicillin-binding protein 2a are the two main mechanisms of resistance in *S. aureus.* The aim of this work was to study the mechanisms of resistance produced by the *S. aureus* strain isolated from *Oreochromis niloticus* (Nile tilapia) during an outbreak. β-Lactamases production was detected by iodometric and clover leaf techniques. The induction of *mecA* gene was done using oxacillin and the gene expression was detected by real time reverse transcription-polymerase chain reaction in the induced isolate and compared with the non-induced one. Also, the studied *mecA* gene was sequenced to check the similarity between the gene of the tested isolate and the published *mecA* genes. Results showed that this isolate produces β-lactamase and *mecA* expression was seven times increased in the case of oxacillin induction. Sequencing results showed 99% identity between the studied gene and the published reference genes. Extensive use of antibiotics in fish farms resulted in the emergence of multidrug resistant staphylococci and this resistance may be induced by the continuous use of some antibiotics.

Key words: Methicillin-resistant *Staphylococcus aureus* (MRSA), *mecA* gene, induction, gene expression.

INTRODUCTION

Outbreaks of bacterial diseases in fish remains one of the most significant limiting factors affecting fish culture worldwide (Gisain et al., 2013) and are among the most important causes of economic losses in cultured tilapia

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(Martins et al., 2008). Staphylococci are among the most widespread opportunist pathogenic bacteria. It has been reported as the causative agent of eye disease on Silver carp (Shah and Tyagi, 1986) and *Channa marulius* (Kumaraiah et al., 1977). Also, methicillin-resistant *Staphylococcus aureus* (MRSA) has been isolated from apparently healthy *Oreochromis niloticus* (Nile tilapia) (Atyah et al., 2010).

MRSA is a major cause of infection worldwide (Rudkin et al., 2014). The emergence and subsequent spread of MRSA from the 1960s to the present, has created clinical difficulties for nosocomial treatment on a global scale (Fuda et al., 2005). Staphylococci have two mechanisms for resistance to beta-lactam antibiotics. One is the production of beta-lactamases, which are enzymes that hydrolytically destroy beta-lactams. The other is the expression of penicillin-binding protein 2a (PBP 2a), which is not susceptible to inhibition by beta-lactam antibiotics. Strains of *S. aureus* exhibiting either beta-lactamase or PBP 2a-directed resistance (or both) have established a considerable ecological niche among pathogenic bacteria (Fuda et al., 2005).

The major mechanism of methicillin resistance in *S. aureus* is the acquisition and expression of *mecA* gene that encodes penicillin-binding protein 2a (PBP2a) (Shang et al., 2010) which has low affinity to beta-lactam antibiotics (Hussain et al., 2000). Regulation of *mecA* expression is controlled by its own regulators, *mecR1* and *mecI*. In the absence of β-lactam antibiotics, *mecA* transcription is repressed by *MecI* bound to its promoter region. Detection of β-lactams by the sensory domains in *MecR1* removes the repression of *mecA* transcription by *MecI*, which leads to *mecA* transcription, PBP2a translation and the expression of methicillin resistance (Rudkin et al., 2014).

Beta-lactam compounds such as penicillin continues to be one of the most frequently used drugs in veterinary medicine (Robles et al., 2014). And it is recommended as the first choice for bacteria that are inherently sensitive to it. In contrast to human isolates, the prevalence of penicillin resistance in staphylococci causing animal diseases can be relatively low and is most commonly due to the production of β –lactamase (Pitkälä et al., 2007).

The aim of this study was to detect the mechanisms of resistance to beta lactam antibiotics in the MRSA isolate causing the outbreak of infection in Nile Tilapia, and to determine the effect of oxacillin on *mecA* expression in the tested MRSA isolate.

MATERIALS AND METHODS

Bacterial isolate

S. aureus isolate was obtained from the kidney of naturally infected Nile Tilapia during an outbreak in Kafr-Elsheikh governorate. Its identification was done by Gram staining, catalase, coagulase tests, cultivation on mannitol salt agar and confirmed by API Staph system.

Antimicrobial susceptibility testing

Susceptibility testing was determined by the disc diffusion method according to Clinical and Laboratory Standards Institute (Wayne 2011) guidelines for 17 antibiotics: chloramphenicol (C), lincomycin (L), cepheadine (CE), gentamicin (G), doxycycline (DO), norfloxacin (NOR), streptomycin (S), apramycin (APR), ampicillin (AMP), ciprofloxacin (CIP), cefoxitine (FOX), oxacillin(OX), amoxicillin-clavulanic acid (AMC), cefotaxime (CTX), ceftriaxone (CRO), ceftazidime (CAZ), cefepime (FEP).

Detection of β-lactamase production by the iodometeric method

Penicillin solution was dispensed in 0.5 ml volume in small test tubes. Test bacteria were removed with a loop from an overnight culture on solid medium and suspended in the Penicillin solution to give a density of at least 10^4 CFU/ml. After one hour at room temperature, two drops of starch indicator was added to the suspension, followed by one drop of Iodine reagent. Positive reaction was indicated by the disappearance of blue color immediately. Persistence of blue color for longer than 10 min constituted a negative test (Miles and Amyes, 1996).

Clover leaf technique

This method was done according to Parvathi and Appala (2000) with some modifications where a Mueller-Hinton agar plate was swabbed with a culture of β lactamase nonproducing strain of *S. aureus* ATCC 25923. An amoxicillin disc (10 units) was placed in the centre of the plate and the test strain was heavily streaked radially outward from the disc to produce growth about 0.25 cm wide. The plate was incubated at 37°C for 18 h and the examined for the presence of clover leaf pattern.

If the strains produced β-lactamase, the zone produced by the β lactamase nonproducing strain was inhibited where the zones of growth of ATCC strain and test strains coincided thus giving rise to a clover leaf pattern. If the test strains did not produce β-lactamase, no clover leaf pattern was produced.

Induction of *mecA* expression by using oxacillin

Induction of *mecA* expression was made according to Hussain et al. (2000) with slight modifications where the bacteria were swabbed on the surface of Mueller-Hinton agar and a disc with 1 µg of oxacillin was placed in the main inoculums. After 18 h incubation, the growth around the disc was used to perform the quantitative reverse transcription polymerase chain reaction (RT-qPCR). RT-qPCR was performed on both the induced and non-induced *S. aureus*, and then the results were compared.

Gene expression by RT-qPCR

RT-qPCR was performed according to method described by Shang et al. (2010) by using the High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, USA). 100 ng of RNA were used in each sample, and MyGenie 32 Thermal Block (BIONEER) was used to perform RT-PCRs.

Real time PCR was performed using SYBR® Green PCR Master Mix (Applied Biosystems, USA) and Stratagene Mx3000P QPCR system (Agilent technologies). The primers used are shown in Table 1.

Table 1. Primers used for gene expression real time PCR (Shang et al., 2010).

Primer	Primer sequence (5–3)	Target gene	Amplicon size (bp)
Mec F	CTCAGGTACTGCTATCCACC	*MecA*	152
Mec R	GGAACTTGTTGAGCAGAGG		
CON F	CCAGCAGCCGCGGTAAT	16S RNA	100
CON R	CGCGCTTTACGCCCAATA		

Sequencing of *mecA* gene

In order to detect the identity of the *mecA* gene of the tested isolate and to search for the presence of mutations that may affect the gene, sequencing of *mecA* gene was performed. The PCR product was purified using PureLink PCR purification kit (Invitrogen Life Technologies, Carlsbad, USA). Automated sequencing reactions were performed with the BigDye terminator cycle sequencing kit and an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster city, USA) using the same primers that were used in the amplification of the *mecA* gene.

Phylogenic analysis

The derived sequence was aligned and compared with those of published reference *S. aureus* strains in the GeneBank using the National Center for Biotechnology Information's BLAST server and the software package (BioEdit v 7.2.5) for multiple sequences alignment and phylogenetic tree construction.

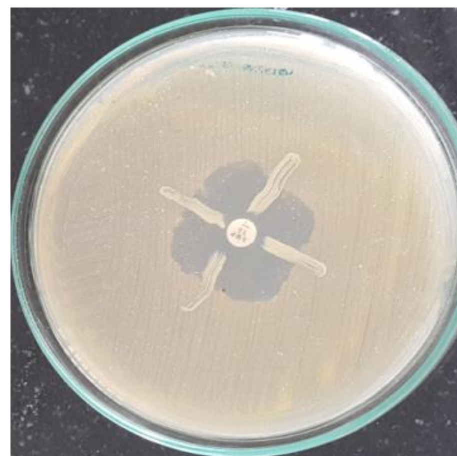

Figure 1. Clover leaf technique showing β lactamase positive result.

RESULTS

The examined isolate was found to be Gram-positive, catalase and coagulase positive. Using API staph system, the isolate was confirmed to be *S. aureus*.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing showed that the bacteria are resistant to all β-lactam antibiotics including ampicillin, cephradine, cefotaxime, ceftriaxone, ceftazidime and cefepime. It was also found to be resistant to oxacillin which indicates methicillin resistance. But they are sensitive to gentamicin, streptomycin, lincomycin, doxycycline, ciprofloxacin, norfloxacin and chloramphenicol.

Beta-lactamase production

The *S. aureus* isolate was found to be β-lactamase producer by the iodometric method where disappearance of the blue color was observed and this was confirmed by the clover leaf method where the zone produced by the control strain was inhibited and the zones of growth of the control strain and test strain coincides thus giving rise to a clover leaf pattern as illustrated in Figure 1.

Expression of *mecA* gene

It was found that oxacillin induced *mecA* expression in the tested MRSA isolate with increase in the expression of 8 folds as compared to the non-induced isolate.

Phylogeny of *mecA* gene

Multiple sequence alignment and phylogenic analysis of *mecA* gene indicated the great similarity (99% identity) between the tested gene sequence and the other *S. aureus* strains published in the Gene bank. This is also shown in the phylogenetic tree (Figure 2).

DISCUSSION

S. aureus is a major human pathogen and is resistant to most commercially available antibiotics. The antibiotic resistance crisis may be attributed to the overuse and misuse of antibiotics (Ventola, 2015). Because of the

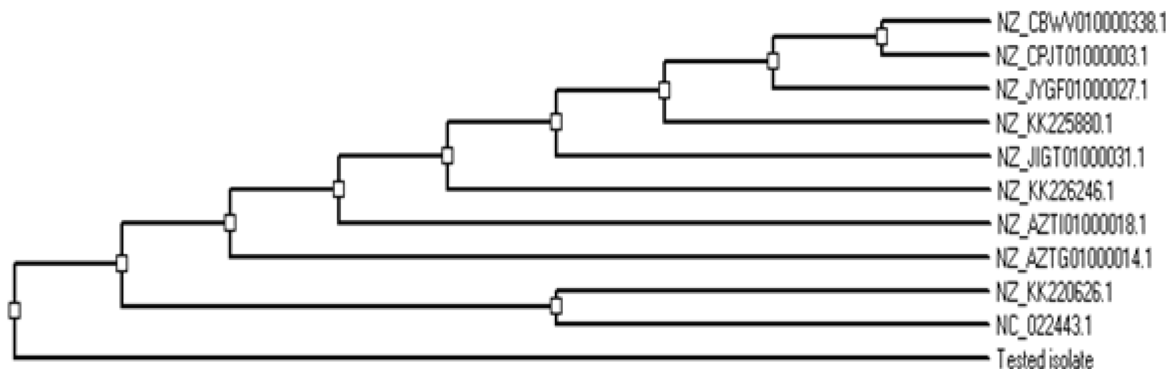

Figure 2. Phylogenetic tree of the *mecA* gene of the tested and reference *S. aureus* strains generated by the software package (BioEdit).

ability of staphylococci to change over time, the MRSA will continue to be a problem in the future. Production of β-lactamases and the expression of penicillin-binding protein 2a (PBP 2a) cause a tremendous problem in medicine because these render *S. aureus* to be resistant to all β-lactam antibiotics (penicillins, cephalosporins and carbapenems) (Lowy, 2003).

In this work, β-lactamase production in *S. aureus* strain isolated from Nile Tilapia fish was studied. The results of antimicrobial susceptibility test showed that the bacteria are resistant to all β-lactam antibiotics and oxacillin. For β-lactamase production, different phenotypic diagnostic methods that are specific and reliable such as the clover leaf and iodometric methods were used (Robles et al., 2014). The iodometric method was considered as an accurate method (Devapriya et al., 2013) while, the clover leaf method was considered as a reliable method for investigating β-lactamase production in staphylococci (Bergan et al., 1997). Whereas Pitkala et al. (2007) estimated that it is more useful in research than in routine use.

Concurrent exposure to antimicrobials may reduce susceptibility to antimicrobial drugs in the major human pathogen, *S. aureus* (Haaber et al., 2015). Here, methicillin resistance and the role of oxacilin in *mecA* gene induction was studied which shows the risk of misuse of antibiotics in increasing the virulence and resistance of staphylococci. Expression of *mecA* and PBP2a gene is known to be inducible by many β-lactam antibiotics with oxacillin being a more potent inducer than methicillin in some strains (Rudkin et al., 2014; Shang et al., 2010). These findings were similar to what is found in this work where there was about 8 folds increase in *mecA* gene expression after subjecting the isolate to oxacillin. The induction of *mecA* is strain dependant as it ranges from 2 to 50 fold increase as reported by Shang et al. (2010).

Cell wall-active antibiotics such as oxacillin, cause induction of a locus in *S. aureus* that leads to elevated synthesis of two methionine sulfoxide reductases (MsrA1 and MsrB). These enzymes reduce methionine sulfoxide and maintain protein integrity and function against oxidative stress. These two proteins have also been shown to have potential roles in bacterial virulence (Singh et al., 2015). There are also a number of studies that have shown that sub-inhibitory concentrations of oxacillin increase rather than decrease staphylococcal virulence by increasing the transcription of toxin genes such as alpha-toxin and Panton-Valentine leucocidin (PVL) in *S. aureus* strains. Oxacillin also induced an overall increase in exoprotein expression levels by MRSA isolates, including alpha-toxin and PVL, revealing that oxacillin has pleiotropic effects on *S. aureus* strains, altering their toxin expression profile (Rudkin et al., 2014).

Findings of this study show that fish play a role in transmission of methicillin-resistant *S. aureus* infection. Also, in concordance with other studies, subjecting the present study MRSA isolate to oxacillin increases the expression levels of *mecA* gene by eight folds.

Results of *mecA* sequencing showed that there is 99% identity between the gene of the tested isolate and those of the published reference strains; this indicated that this fish pathogen may be a source of infection to human who handle them or use them as food which may lead to intoxication. Finally it can be concluded that the misuse of antibiotics may result in the emergence of highly resistant bacteria which may be a health hazard to fish and may also be transmitted to humans.

Conflict of Interests

The authors have not declared any conflict of interests.

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