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Full Length Research Paper

Microbiological evaluation and development of quality index method (QIM) scheme for farmed pintado fish (*Pseudoplatystoma corruscans*)

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Experiments were conducted to evaluate the microbiological quality and develop a quality index method (QIM) scheme for farmed pintado fish (*Pseudoplatystoma corruscans*), with estimation of shelf life. Microbiological analyses showed that two samples of pintado fish were unacceptable for human consumption due to the presence of *Salmonella* spp. The presence of *Staphylococcus aureus* in all six samples of pintado can be related with poor hygiene during handling, since *S. aureus* is a bacterium known to be carried by food handlers. A QIM scheme based on a total of 16 demerit points was developed. The QIM scheme proved to give a good description of the changes in whole fresh pintado during ice storage. The quality index (QI) evolved linearly with storage time in ice (R^2 = 0.97). Results indicated that the shelf life of pintado fish (whole and gutted) stored in ice is about 13 days.

Key words: Quality index method, *Pseudoplatystoma corruscans*, freshwater fish, fish quality, shelf life, sensory analysis.

INTRODUCTION

The native freshwater fish species of Brazil are numerous and many of these species are important for commercial production in South America, with good potential for aquaculture. The pintado (*Pseudoplatystoma corruscans*), also known as surubim, is a carnivorous species native of the Paraná River and São Francisco River basins. Pintado has a great commercial potential due to its size (it may reach 50 kg) and also due to its clear and delicate flesh, which presents few inter-muscle bones (Campos et al., 2006; Tanamati et al., 2009).

Due to over exploitation of the stocks, wild populations of pintado have been decreased in the last decade (Martino et al., 2002). Nowadays, some commercial farms are achieving good growth rates in captivity. The farming

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of pintado is growing in Brazil; however the production is still modest and concentrated in the Centro-Oeste region (Campos et al., 2006; Martino et al., 2002). According to IBGE (2013), the Brazilian production of pintado in 2013 was 15.715 tons and the Centro-Oeste region (Mato Grosso, Mato Grosso do Sul and Goiás) was responsible for 82.9% of the production.

Fresh fish are susceptible to rapid spoilage. The high water and protein content and neutral pH of fresh fish renders them sensitive to microbial attack (Boziaris et al., 2013; Gram and Dalgaard, 2002). Considering that fresh fish spoil mainly owing to microbial action, the microbiological evaluation is essential in determining the quality and the safety of fish (Gram and Dalgaard, 2002; Teklemariam et al., 2015).

The main quality attributes of fish are safety, nutritional characteristics and freshness. The most common methods to evaluate freshness of fish are the sensory methods. Evaluation procedures for raw fish should be simple to apply and specific for particular fish species. The quality index method (QIM) is a grading system for estimating the freshness of fish, based on objective evaluation of the main sensory attributes of each fish species using a demerit points scoring system. The QIM scheme is designed in a way that the quality parameters of very fresh fish receive zero points. Deterioration progresses of fish with storage time in ice receive the maximum of 3 demerit points. Therefore, higher scores are given as storage time progresses. A quality index (QI) is obtained by the sum of demerit points, which allows evaluating fish quality and predicting the shelf life of the species (Bernardi et al., 2013; Lanzarin et al., 2016; Sveinsdottir et al., 2003).

QIM schemes have been developed for a number of fish species including: farmed Atlantic salmon (Sveinsdottir et al., 2003), fresh cod fillets (Bonilla et al., 2007), Brazilian corvine (Teixeira et al., 2009), Brazilian sardines (Andrade et al., 2012), Brazilian Amazonian Pintado (Lanzarin et al., 2016) and others. The aim of this study was to determine the microbiological quality and to develop a quality index method (QIM) scheme for farmed pintado fish (*P. corruscans*), with estimation of shelf life.

MATERIALS AND METHODS

Samples used for microbiological analyses

Samples were collected from six different supermarkets in the city of Brasilia, DF, Brazil, from September to December 2015. The samples of pintado stored in ice were represented by six whole fish gutted and cut in pieces, prepared at the time of purchase in the supermarkets. Fresh pintado was from aquaculture and the samples had an average weight between 2.3 and 2.7 kg. The time needed to transport samples from supermarkets to the laboratory was 20 to 40 min. The samples were transported in a cool box and microbiologically examined within maximum 1 h of sampling. The samples were analyzed in triplicate and results were expressed as mean.

Microbiological analyses

All the samples were analyzed for each of the following micro-organisms or microbial groups: total aerobic mesophilic and psychrotrophic bacteria, total and thermotolerant coliforms, *Staphylococcus* spp. and *Salmonella* spp. An amount of 25 g of each sample was diluted on 225 mL of 0.1% peptone water. Samples were homogenized and an initial 10^{-1} dilution was obtained. Then, serial dilutions of the homogenates were prepared in 0.1% peptone water (up to 10^{-5}).

For total aerobic mesophilic and psychrotrophic bacteria counts, serial dilutions of the samples were surface plated in plate count agar (PCA) (HiMedia, USA), following incubation at 37°C for 24 h for mesophilic bacteria and 8-10°C for 7 days for psychrotrophic bacteria. The results were expressed by colony forming unit per gram (CFU g^{-1}).

For detection of total and thermotolerant coliforms, 1 mL of each dilution was transferred to three-tube series containing lauryl sulfate tryptose (LST) (HiMedia, USA) with Durham tubes in its interior. Total coliforms were enumerated in Brilliant Green Bile Broth 2% (HiMedia, USA), incubated at 37°C for 24 h and thermotolerant coliforms were determined in *E. coli* broth (EC) (Acumedia, USA) incubated at 45°C for 24 h. The results were expressed by most probable number per gram (MPN g^{-1}).

For *Staphylococcus aureus* counts, serial dilutions of the samples were surface plated in Mannitol Salt Agar (HiMedia, USA), following incubation at 37°C for 48 h. The colonies were counted and sub-cultured in Mannitol Salt Agar tubes. The characteristic colonies of *S. aureus* (yellow colonies with yellow zones, mannitol-fermenting) were stained by Gram's method to confirm Gram-positive cocci. The colonies of *S. aureus* were further confirmed through molecular analyses.

The detection of *Salmonella* spp. was carried out with pre-enrichment in lactose broth and incubation at 37^{-1} C for 24 h. Subsequently, 1 mL of this medium was inoculated on Selenite cystine broth that was incubated at 37^{-1} C for 24 h. Further, Salmonella Shigella Agar (SS) (HiMedia, USA) was streaked with sterile loops carrying inoculums taken from Selenite cystine broth. The plates were incubated at 37^{-1} C for 24 h in order to isolate characteristic colonies of *Salmonella* spp. Triple sugar iron (TSI) (HiMedia, USA) was used for presumptive confirmation of colonies that were further confirmed through molecular analyses.

Molecular analyses

The potential pathogenic bacteria *S. aureus* and *Salmonella* spp. were identified using the technique of polymerase chain reaction (PCR). For identification of *S. aureus* the primers CoA-F and CoA-R (Table 1) (Life Technologies, Brazil) specific for the coagulase gene (coa gene) of *S. aureus* were used. For identification of *Salmonella* spp. the primers InvA-F and InvA-R (Life Technologies, Brazil) (Table 1) specific for the invasion A gene (invA gene) of *Salmonella* spp. were used.

For DNA extraction, the NucleoSpin Plasmid® kit (Macherey-Nagel Inc.; Germany) was used, following the manufacturer's instructions. Extracted DNA was stored at -20°C. PCR was performed in a reaction mixture of 25 µl final volume containing 2.5 µl of PCR buffer; 0.7 µl of $MgCl_2$; 1.5 µL of dNTP (2,5 mM); 0.5 µl of Taq DNA polymerase; 1.5 µL of each primer forward and reverse and 18.3 µl of Milli-Q water.

PCR amplification was performed with an initial denaturing step at 95°C for 1 min, followed by a 35-cycle reaction (95°C for 1 min

Table 1. Primers sequence and size of amplified products of PCR.

Primer	Sequence 5´- 3´	Amplified product	Species
CoA-F foward	GATCTTCGCGTGATACGTCA	303 bp	*S. aureus*
CoA-R reverse	GTTCGTGCAATGTTTTGTCC		
InvA-F foward	CATTGGTGATGGTCTTGTCG	298 bp	*Salmonella* spp.
InvA-R reverse	CTCGCCTTTGCTGGTTTTAG		

Table 2. Counts of mesophilic and psychrotrophic bacteria, enumeration of total and thermotolerant coliforms, counts of *S. aureus* and detection of *Salmonella* spp. in the samples of pintado fish.

Samples	Mesophilic bacteria (log CFU/g)	Psychrotrophic bacteria (log CFU/g)	Total coliforms (log MPN/g)	Thermotolerant coliforms (log MPN/g)	*S. aureus* (log CFU/g)	*Salmonella* spp.
1	5.0 ± 0.03	5.3 ± 0.07	1.0 ± 0.01	0.6 ± 0.06	2.0 ± 0.01	Positive
2	6.8 ± 0.11	8.0 ± 0.29	2.4 ± 0.01	ND	2.3 ± 0.01	Positive
3	3.9 ± 0.62	6.8 ± 0.44	0.8 ± 0.01	ND	2.8 ± 0.04	Negative
4	5.3 ± 0.26	6.3 ± 0.06	1.8 ± 0.05	0.8 ± 0.01	2.3 ± 0.01	Negative
5	3.0 ± 0.48	4.2 ± 0.09	1.4 ± 0.01	0.8 ± 0.01	2.8 ± 0.08	Negative
6	4.3 ± 0.28	6.4 ± 0.06	1.2 ± 0.01	1.2 ± 0.07	2.3 ± 0.34	Negative

Results are reported as means ± standard deviation of triplicate measurements; ND: not detected.

and 60°C for 1 min). A final extension step was undertaken at 72°C for 1 min. All thermal cycling reactions were performed with Techne TC-512 thermal cycler (Bibby Scientific Inc., USA). Both negative and reagent controls were included in each PCR run. The reagent control consisted of all PCR components except the template DNA.

The entire amplified DNA was separated by electrophoresis at 100 V for 50 min in 1.5% (w/v) agarose gel and stained with ethidium bromide. Gels were visualized under UV light. A 100 bp DNA ladder was used as a molecular weight marker.

Samples used for quality index method

The experiments for quality index method were performed between November, 2015 and April, 2016. Fresh pintado was from an aquaculture farm located in the state of Tocantins, Brazil. Three experiments for quality index method were performed and then 18 fish were used in total. For each experiment, 6 whole and gutted fish were kept iced and boxed in a refrigerator set at 8°C. These boxes had perforated bottoms, which allowed drainage of melted ice. Thus, ice was added on a daily basis to replace the melted fraction. Direct contact of ice with fish skin was the option chosen. The samples had an average weight of 2.3 and 2.7 kg.

Quality index method (QIM)

To develop the quality index method, the first experiment included the preparation of the preliminary QIM scheme for sensory evaluation of fresh pintado fish. Two experts observed the 6 samples of pintado after 1, 3, 5, 7, 9, 11, 13, 15 and 17 days of storage in ice. All observations were conducted under standardized conditions at room temperature. The first experiment was developed to find the characteristics that change clearly with time, necessary for the first draft of the QIM table. Major sensory changes were selected among the listed attributes and described in detail in preliminary scheme. A score of 0 to 3 demerit points was given for every change of evaluated parameter.

The second experiment of the QIM development included training of the panelists and testing of the preliminary scheme. The panel consisted of 6 panelists and the preliminary QIM table and instructions on how to perform the correct evaluation was used to train the panel. For testing the preliminary scheme, a total of 6 fish were examined after 1, 3, 5, 7, 9, 11, 13, 15, 17 and 19 days of storage in ice. All suggestions of improvements by the panel members, in the evaluation, were included in the final scheme. During this period, microbiological analyses were performed with count of total mesophilic bacteria.

The third experiment was used to validate the QIM scheme developed in the previous evaluations. The trained panel members evaluated a total of 6 fish after 1, 3, 5, 7, 9, 11, 13, 15, 17 and 19 days of storage in ice. The QIM scheme for whole fresh and gutted pintado listed quality attributes for appearance, eyes, gills and abdomen area and described how they change with storage time. Scores were given for each quality attribute ranging from 0 to 3 demerit points. The overall sum of demerit points was designated as quality index (QI) and could reach a total of 16 demerit points. During this period, microbiological analyses were performed with count of total mesophilic bacteria.

RESULTS AND DISCUSSION

Microbiological and molecular analyses

The results of microbiological analyses are shown in Table 2. The count of mesophilic bacteria ranged from

3.0 to 5.0 log CFU/g and the count of psychrotrophic bacteria ranged from 4.2 to 6.8 log CFU/g in most samples. Only one sample of pintado fish (sample 2) had a higher count of 6.8 log CFU/g for mesophilic bacteria and 8.0 log CFU/g for psychrotrophic bacteria.

The International Commission on Microbiological Specifications for Foods (ICMSF, 1986) recommends the maximum acceptable limit of 7 log CFU/g for total count of bacteria in fresh fish. An increase in total count to levels in excess of 6 log CFU/g is usually indicative of long storage at chilling temperatures or temperature abuse prior to freezing. Thus, total count of bacteria is indicative of general quality and to a lesser extent of handling and storage procedures (ICMSF, 1986).

In this study, most samples (excluding sample 2) showed acceptable counts of total mesophilic and psychrotrophic bacteria. Delbem et al. (2012) found similar results and reported average populations of 3.3 to 4.4 log CFU/g of mesophilic bacteria in samples of pintado fish acquired in the city of Corumbá, Mato Grosso do Sul, Brazil.

All samples of pintado presented low numbers of total and thermotolerant coliforms. The low numbers of total and thermotolerant coliforms in samples indicates a good quality of the water where fish inhabit. If the fish were caught in polluted waters, Enterobacteriaceae (total coliforms) could be the mainly spoilers. Fish do not usually carry these organisms generally considered to be typical of the mammalian microflora, including *Escherichia coli* (thermotolerant coliforms). The presence of human enteric organisms in flesh of fish is clear evidence of faecal contamination in waters (Ampofo and Clerk, 2003; Guzmán et al., 2004).

Despite the inexistence in Brazilian legislation (Brasil, 2001) of microbiological limits for thermotolerant coliforms or *E. coli* in fresh fish, the maximum value suggested by ICMSF (1986) for *E. coli* is 2.7 CFU/g. Delbem et al. (2012) found similar results and reported average enumeration of 1.3 log MPN/g of thermotolerant coliforms in samples of pintado acquired in the city of Corumbá, Mato Grosso do Sul, Brazil.

On the other hand, *Salmonella* spp. was found in two samples of pintado fish (samples 1 and 2). *Salmonella* is not a component of the normal flora of fish, thus its contamination is consequence of fecal contamination through polluted water, infected food handlers or cross-contamination during production or transport (Carrasco et al., 2012). According to Brazilian legislation (Brasil, 2001), the presence of *Salmonella* in fresh fish is unacceptable (absence/25 g) and may represent a risk for consumers. Therefore, samples 1 and 2 were unacceptable for human consumption.

Onmaz et al. (2015) reported 5 samples of a total of 100 fish collected in Turkey contaminated with *Salmonella* spp. It was concluded that the prevalence of *Salmonella* spp. is frequently attributed to poor hygienic practices during handling and transportation from landing centers to fish markets. After molecular analyses, there was specific amplification of gene region *invA* in samples 1 and 2. According to Kumar et al. (2006), the invA gene (responsible for invasion protein A), recognizes the *Salmonella* genus. The invA gene is an important virulence factor of *Salmonella* spp. It is responsible for invasion of epithelial host cells and has been reported to be present in all salmonellas.

All samples of pintado were contaminated with *S. aureus* (confirmed by PCR). The maximum value allowed by the Brazilian legislation (Brasil, 2001) for *S. aureus* in fresh fish is 3 log CFU/g. Although, all samples were in accordance with the Brazilian legislation, it is important to underline that *S. aureus* may occur on fish usually only in low numbers and its contamination probably is consequence of food handlers (ICMSF, 1986). Therefore, good hygiene practices must be implemented by producers in order to prevent *S. aureus* contamination. Gatti Junior et al. (2014) reported counts of *Staphylococcus* spp. in tilapia's fillet ranging from 2.0 to 4.0 log CFU/g and concluded that this contamination was a consequence of improper and unsanitary handling.

The coagulase gene (coa) has been reported to be specific for confirmation of *S. aureus* and the coagulase protein is an important virulence factor of *S. aureus*. Others studies (Gandra et al., 2005; Motta et al., 2001; Shopsin et al., 2000) used the coa gene to confirm *S. aureus* in samples. Gandra et al. (2005) used the coa gene for the differentiation between *S. aureus* and *Staphylococcus intermedius* and *Staphylococcus hyicus.* Motta et al. (2001) reported that all *S. aureus* isolates from fresh milk were positive for the presence of the coa gene determined by PCR.

Quality index method (QIM)

The QIM scheme presented (Table 3) proved to give a good description of the changes in whole fresh pintado during ice storage. The final QIM scheme developed for the farmed pintado fish included 8 parameters, grouped in 5 main attributes and had a total of 16 demerit points. Attributes were defined as appearance, eyes, gills, flesh and abdomen area. During the development of the scheme, the rotten odour was changed to very sour and for the attribute eyes, scores were added to 3 points because sunken shape eyes were observed.

At the beginning of the storage time (0-7 days), the pintado was very fresh (QI = 0-1.2). From the seventh day of storage in ice, QI average was 3.5 and it has been possible to observe the gills with dark red colour, the skin with darker colour and the eyes with flat shape, black pupils and slightly opaque cornea, but the odour still remained neutral.

The maximum storage life of pintado was 13 days in ice. During this period, the average QI was between 4.5 and 7.5 demerit points. Various sensory changes were

Table 3. Scheme of the quality index method (QIM) proposed for farmed pintado fish (*Pseudoplatystoma corruscans*).

Quality attribute	Parameter	Description	Points
Appearance	Skin aspect	Intact skin with well-defined spots	0
		Skin with darker color	1
		Skin with darker color and less defined spots	2
	Odour	Fresh, neutral	0
		Less fresh, slightly sour	1
		Sour	2
		Very sour	3
	Skin mucus	Absent	0
		Transparent, clear	1
	Skin dorsal colour	Very white	0
		Yellowish, yellow below the mouth	1
Eyes	Eyes aspect	Convex shape, black bright shiny pupils and transparent cornea	0
		Flat shape, black pupils and slightly opaque cornea	1
		Little sunken shape, pupils and cornea opaque	2
		Sunken shape, pupils and cornea opaque	3
Gills	Colour	Bright red	0
		Dark red	1
		Brown, pale brown	2
Flesh	Flesh Aspect	Bright, light pink colour, remember chicken breast meat, firm texture	0
		Opaque, rusty pink colour, remember chicken thigh meat, firm texture	1
		Opaque, rusty pink colour, remember chicken thigh meat, softened texture with skin fat crumbling	2
Abdomen area	Abdomen aspect	Bright, light pink colour, fresh odour	0
		Opaque, rusty pink colour, fresh or less fresh odour	1
		Opaque, rusty pink colour, sour odour	2
Quality index (QI)			0-16

observed as the beginning of sour odour upon opening the box (slightly sour) which dissipated completely when handling the fish, the gills with dark red colour, the eyes with little sunken shape, pupils and cornea opaque and the abdomen starting to get old aspect (opaque, rusty pink colour).

After 15 days of storage in ice, the odour was increasingly characterized by sour and this indicated that pintado was in the end of shelf life and acceptability. After 17 days of storage in ice, pintado had already been rejected, with sour odour and flesh with old aspect (opaque, rusty pink colour). Between 15 and 19 days of storage in ice, typical sensory changes of the deterioration progressed rapidly, with very sour odour quite noticeable in the abdomen area, the flesh was with softened texture and fat crumbling and the dorsal area was with a yellowish colour in the area between the mouth and gills.

Similar results for the proposition of the QIM scheme were found by Lanzarin et al. (2016) with maximum score of 18 points for gutted Amazonian Pintado (*Pseudoplatystoma fasciatum* x *Leiarius marmoratus*). In other studies, Sveinsdottir et al. (2003) and Teixeira et al. (2009) developed a QIM scheme based on a total of 22 demerit points for Atlantic salmon and Brazilian corvine, respectively. The differences between the QIM schemes show that this is a specific method that considers the differences observed in the deterioration process for the distinct species.

Lanzarin et al. (2016) described that the quality attributes that most influenced the QIM protocol of fresh gutted Amazonian Pintado were abdominal odour and abdomen colour (yellowish flesh). In this study, these characteristics were observed when pintado was rejected, between 15 and 19 days, with very sour odour in the abdomen area and the dorsal area with a yellowish colour. In the final developed QIM scheme, there was a linear relationship with high correlation (R^2=0.97) between the average QI with storage time in ice (Figure 1).

Table 4 presents the counts of mesophilic bacteria in

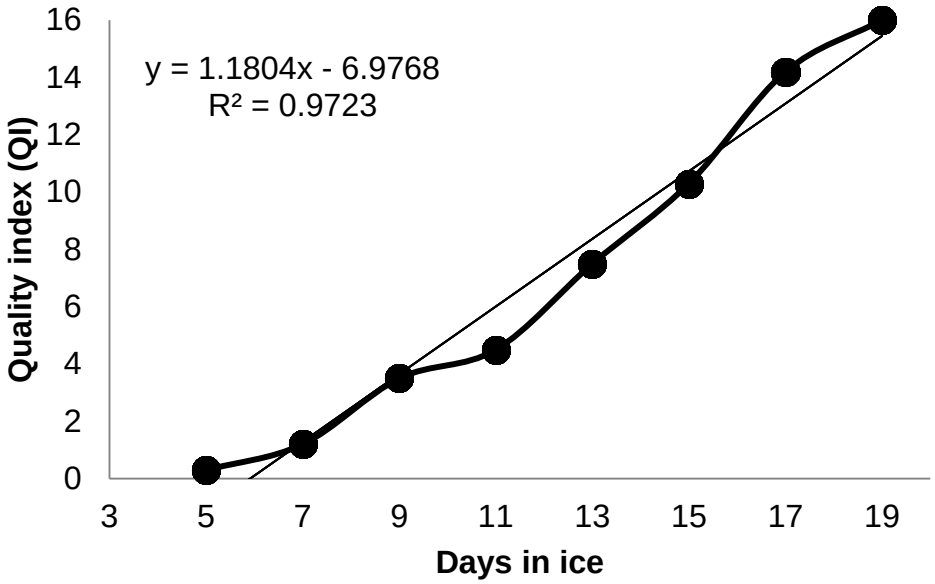

Figure 1. QI linear correlation with ice storage days of the 16 demerit points for QIM scheme proposed for farmed pintado fish (*Pseudoplatystoma corruscans)*

Table 4. Count of mesophilic bacteria in pintado fish stored in ice.

Storage days in ice	Mesophilic bacteria count (log CFU/g)
1	4.2 ± 0.01
3	4.3 ± 0.02
7	4.3 ± 0.01
9	4.9 ± 0.03
11	5.2 ± 0.01
13	6.0 ± 0.01
17	6.5 ± 0.01

Results are reported as means ± standard deviation of three measurements.

the samples of pintado fish stored in ice for 19 days. An initial bacterial flora of around 4 log CFU/g remained constant along the first 7 days in ice, while fish was still fresh, with good quality. Between 9 and 11 days of storage in ice, when the fish was less fresh, the mesophilic count increased to 5 log CFU/g. The maximum storage life of pintado was 13 days in ice, when the mesophilic bacteria counts were 6.0 log CFU/g. In 17 days of storage in ice, when the fish was rejected, the mesophilic bacterial count reached 6.5 log CFU/g. According to Barbosa et al. (2002), the total bacterial count just after catch fish or at the start of storage is variable between 1 and 4 log CFU/g, but at the time of sensorial rejection, it was between 6 and 8 log CFU/g, levels which are in accordance with the counts of microorganisms obtained in the present work.

Lanzarin et al. (2016) estimated that the shelf life for ice stored gutted Amazonian Pintado was 12 days, when the mesophilic bacteria counts were 6.5 log CFU/g. At 14 days of storage, the population of these bacteria increased to 7.1 log CFU/g and Amazonian Pintado was considered unacceptable for consumption

In conclusion, microbiological analyses showed that two samples of pintado were unacceptable for human consumption due to the presence of *Salmonella* spp. The other four samples were acceptable for consumption, although the presence of *S. aureus* in all samples can be related with poor hygiene during handling. The QIM scheme has proved to be effective for assessing the quality of fresh water pintado fish (whole and gutted) stored in ice, indicating that its shelf life is about 13 days.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Antibiotic resistance patterns of lactic acid bacteria isolated from Nigerian grown salad vegetables

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The reports on some foodborne lactic acid bacteria (LAB) possessing antibiotic resistance (AR) genes on mobile genetic elements are on the increase. In Nigeria, such information is rare. This study was therefore designed to determine the presence and locations of AR genes in LAB isolated from locally grown salad vegetables. The LAB used in this study were previously isolated from Nigerian grown cabbage, carrot, cucumber and lettuce and identified by partial sequencing of their 16S rRNA gene. The AR and integrons (*intI*1, 2, 3) genes were detected using polymerase chain reaction after phenotypic agar disc diffusion assay of 20 antibiotics. Extraction and curing of plasmids were performed using standard methods. Univariate analysis was performed to determine resistance to ≥ 2 antibiotics, while multinomial logistic regression was conducted to determine association of resistance patterns with vegetable sources/ types and LAB strains at 95% confidence interval (CI). The entire LAB were phenotypically resistant to ≥2 antibiotics, while uncultured Solibacillus clone RBL-135 was resistant to all and possessed the 454 bp vancomycin (*van*X) gene on chromosomes. Three others, *Lactobacillus plantarum* YML 007 (lettuce), *Lactobacillus plantarum* TCP 008 (cabbage) and *Weissella cibaria* PON 10339 (carrot), also amplified this gene while *Weissella confusa* SJL 602 (lettuce) amplified resistance gene for beta lactam (*bla*Z). This gene (*bla*Z) was also detected in three other LAB but with size corresponding to 500 bp. None of the tetracycline ribosomal protection protein, *tet*(M), (S), (W), efflux *tet*(K), (L), aminoglycoside acetyltransferase and phosphotransferase encoding gene, *aac*(6^1)- *aph*(2^1), integrons (*intI* 1,2,3) were detected. The plasmid cured LAB exhibited same resistance patterns as their wild derivatives. The naturally occurring LAB in the study vegetables are phenotypically multidrug resistant, a few possessing *van*X and *bla*Z resistance genes on chromosomes. Hence, they lack potentials to transfer AR through plasmids and integrons.

Key words: Antibiotic resistance, lactic acid bacteria, salad vegetables, polymerase chain reaction, 16S rRNA, plasmids.

INTRODUCTION

Lactic acid bacteria (LAB) are a group of beneficial microbes that are Generally Regarded As Safe (GRAS)

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and this has qualified them suitable as probiotics and starter cultures (Strom et al., 2005; Gerez et al. 2009). They are residents of different niches such as fermented foods/dairy products, vegetables, meat, gastrointestinal tracts of humans and animals.

The introduction of antibiotics in human clinical medicine and animal husbandry is one of the greatest achievements of 20th century (Aarestrup, 2005). This was however doused by different reports of antibiotics resistance (AR) by clinically important bacteria and even commensals. Aquilanti et al. (2007) reported the spread of AR in commensal bacteria, creating large reservoirs of AR genes in nonpathogenic bacteria that are linked to the food chain. Most investigations before now have focused on clinically important bacteria (Gevers et al., 2003a; Rizzotti et al., 2009), while information on LAB AR determinants are largely scarce (Jacobsen et al., 2007). In the past decade, attention is being given to this subject. For instance, there has been increasing evidence that points at a crucial role of foodborne LAB as reservoir of potentially transmissible AR genes and this was as a result of continuous assessment of fermented foods for presence of AR strains by several European countries (Witte, 2000; Teuber, 2001; Hammerum et al., 2007; Toomey et al., 2010). Phenotypic and molecular characterization of 121 strains of *Lactobacillus paracasei* isolated from Italian dairy and meat products demonstrated the presence of tetracycline [tet(M), tet(W)] and/or erythromycin resistance erm(C) in different AR isolates (Comunian et al., 2009). Toomey et al. (2010) also reported transfer of *tet*(M) resistance gene from *L. plantarum* to *Lactococcus lactis* BU-2-60 and *E. faecalis* JH2-2. The resistances of food borne LAB to glycopepetide, vancomycin and the β- lactams have been reported at different times although the reports on the latter are scanty. Mobile elements such as plasmids, transposons and integrons are important drivers in bacterial horizontal gene transfer (HGT) and these constitute AR genes with intra and inter- specie transfer of genetic material (Alekshun and Levy, 2007; van Reenen and Dicks, 2011; Santagati et al., 2012). This is dangerous if it occurs in food borne LAB as the AR genes can easily be transferred to potential pathogens in the gastrointestinal tract of man. In Nigeria, regulation on antibiotics administration both as growth promoter in animal husbandry and in clinical management is lacking. The methods of cultivation of salad vegetables using animal dung/human faeces as manure on one hand and the raw consumption of these vegetables on the other encourage transfer of bacteria which carry AR genes; hence there is need to initiate a sustainable biosafety surveillance in terms of AR determinants of the resident LAB in these vegetables.

This study which to the best of the authors' knowledge, is the first in Nigeria, investigated the linkage of mobile genetic elements, plasmid and integrons with AR in LAB isolated from locally grown salad vegetables.

MATERIALS AND METHODS

Sources and identification of LAB

The lactic acid bacteria used in this study were previously isolated from Nigerian grown salad vegetables, cabbage, carrot, cucumber and lettuce procured from 12 different vegetable markets in Lagos, Nigeria. These markets are located at Agboju, Festac, Idi-Araba, Isolo, Ketu, Mafoluku, Mile 12, Mushin, Oja- Oba, Oke-Odo, Oshodi and Yaba. They were identified with combinations of phenotypic and partial sequencing of 16S rRNA gene (Bamidele et al., 2014). These comprised of *Lactobacillus* spp. (8), *Weissella* spp. (3), *Pediococcus* spp. (3), Uncultured *Solibacillus* (1) and *Enterococcus durans* (1) (Table 1).

Antibiogram of LAB

Twenty antibiotics (Table 2) selected based on the previous works done on LAB and clinical relevance, were employed in the susceptibility assays against the LAB. The antibiotics were aseptically placed with the use of disc dispenser on the Lactobacillus susceptibility test medium (LSM) which was previously seeded with overnight growth of LAB isolates that have been diluted to make 0.5 Mac- Farland standard (10^8 CFU/ml). The LSM is made of the combination of MRS (10%) and Isosensitest (90%) agar (Klare et al., 2007). This was done according to Clinical and Laboratory Standards Institute (CLSI 2007) guidelines. The culture was incubated for 24 h after which zones of inhibition were measured in millimeter (mm) and absence of zones taken as resistance.

PCR detection of resistance genes

The primers for specific genes were synthesized by Inqaba Biotech, South Africa. The annealing temperatures for the reaction were calculated based on the melting temperatures (Tm) of each set of primers (that is, forward, F and reverse, R). The amplification was carried out in thermo cycler (vapo. protect, Eppendoff) using AccuPower PCR premix mastermix (Bioneer, USA) added to 3 µl of template DNA. The mixture was made up to 20 µl with distilled water according to the manufacturer's instructions. The primers, genes and PCR conditions are as shown in Table 4.

Plasmid extraction

This was done according to the modified methods of Klaenhammer (1984). Briefly, overnight culture of LAB was suspended in 200 µl of 25% w/v sucrose, 50 mM Tris HCl, 5 mM EDTA (STE buffer, pH 8.0) containing 5 mg/ml lysozyme (Carlroth, Germany) and 10 Uml^{-1} mutanolysin (Sigma, USA). This was incubated at 37°C for 30 min and 250 µl STE buffer (pH 10.5 adjusted by 10 N NaOH) containing SDS (6% w/v) was added followed by mixing (inverting tubes) 10 times. All these were incubated at 62°C for 30 min and cooled to room temperature. On addition of 300 µl potassium acetate (3 M), the mixture was vortexed and later incubated at -20°C for 10 min. This was followed by centrifugation (10,000 rpm for 15 min) after which the supernatant was transferred into sterile Eppendoff tube. The supernatant was extracted with 500 µl phenol/chloroform/ isoamylalcohol (25:24:1) and centrifuged. After centrifugation, the aqueous phase was transferred into sterile Eppendoff tube and precipitated with isopropanol (equal volume), vortexed and centrifuged for 10 min. The supernatant was decanted and pellet dried in air. The dried pellet was dissolved in 50 µl Tris HCl, EDTA (TE) buffer.

Table 1. Summary of LAB and their source vegetables.

LAB	Cabbage (n)	Carrot (n)	Cucumber (n)	Lettuce (n)	Total (n)
W. confusa	8	2	3	7	20
W. cibaria	4	5	2	2	13
W. paramesenteroides	-	-	1	-	1
Lactobacillus spp.	1	-	-	-	1
L. fermentum	4	1	5	2	12
L. plantarum	7	2	1	5	15
L. reuteri	-	1	-	-	1
L. paralimentarius	-	1	-	-	1
L. brevis	-	2	1	-	3
L. johnsonii	-	-	1	1	2
L. vaginalis	-	-	-	1	1
P. pentosaceus	2	1	6	3	12
P. dextrinicus	1	-	-	-	1
P. acidilactici	-	-	5	2	7
Uncultured solibacillus	-	-	1	-	1
E. durans	-	-	-	1	1

Table 2. The antibiotics used for LAB antibiogram/concentrations.

Antibiotic (code)	Concentration (µg)	Source
Sulphamethoxazole/ Trimethoprim (SXT)	25	Oxoid, UK
Rifampicin (RD)	5	,,
Ciprofloxacin (CIP)	5	,,
Nalidixic acid (NA)	30	,,
Levofloxacin (LEV)	5	,,
Cephalothin (KF)	30	,,
Amoxycillin (AML)	10	,,
Cefocitin (FOX)	30	,,
Ceftriaxone (CRO)	30	,,
Vancomycin (VA)	5	,,
Clindamycin (DA)	5	,,
Imipenem (IPM)	10	,,
Cloxacillin (OB)	5	,,
Cotrimozaxole (COT)	25	Abtek, UK
Chloramphenicol (CHL)	10	,,
Tetracycline (TET)	10	,,
Streptomycin (STR)	10	,,
Augmentin (AUG)	30	,,
Gentamycin (GEN)	10	,,
Erythromycin (ERY)	5	,,

Agarose gel electrophoresis

The PCR products, (5µl) was loaded alongside a 100 bp marker (Solis biodyne, Estonia) on a 1% agarose stained with ethidium bromide. For plasmid, the dissolved pellet (5 µl) was loaded alongside a Lambda DNA/ HindIII marker (Thermo Scientific, USA) on a 0.8% agarose stained with ethidium bromide. This was run for 1 h at 100 V after which they were viewed under UV in a photodocumentation system (Clinix, China).

Plasmid curing experiment

Thirty LAB possessing plasmid DNA, showing phenotypic resistance to at least 2 antibiotics were selected and grown microaerophilically for 72 h at 40°C in sub-lethal dose (75 µg/ml) of acridine orange (BDH) in MRS broth. This dose was arrived at after subjecting them individually to different doses (25, 50, 75 and 100 µg/ml).

The post exposure curing was done by subculturing LAB into

Table 3. Antibiotic susceptibility/ resistance patterns of LAB spp.

Antibiotics/conc (µg)	LAB spp					
	Lactobacillus spp. (n)		*Pediococcus* spp. (n)		*Weissella* spp. (n)	
	R	S	R	S	R	S
Sulphamethoxazole/ Trimethoprim (SXT-25)	14	10	10	4	15	10
Rifampicin (RD- 5)	2	19	2	11	0	22
Ciprofloxacin (CIP- 5)	12	8	4	5	6	14
Nalidixic acid (NA- 30)	19	1	9	0	19	2
Levofloxacin (LEV- 5)	0	7	1	3	2	16
Cephalothin (KF- 30)	0	9	0	4	0	17
Amoxycillin (AML- 10)	9	14	3	12	6	19
Cefoxitin (FOX- 30)	4	15	4	9	3	19
Ceftriaxone (CRO- 30)	22	0	14	1	24	1
Vancomycin (VA- 5)	22	1	14	1	21	2
Clindamycin (DA- 5)	2	20	1	12	4	21
Imipenem (IPM- 10)	0	23	0	15	0	25
Cloxacillin (OB- 5)	22	2	16	0	27	1
Cotrimozaxole (COT- 25)	17	3	11	4	15	9
Chloramphenicol (CHL- 10)	3	17	1	4	1	23
Tetracycline (TET- 10)	10	9	8	7	5	19
Streptomycin (STR- 10)	9	11	8	7	10	14
Augmentin (AUG-30)	12	8	10	5	14	10
Gentamycin (GEN-10)	1	19	0	15	1	23
Erythromycin (ERY- 5)	2	18	0	14	1	23

[R]Resistant, [S]Sensitive. Uncultured *Solibacillus* was resistant to all the tested antibiotics.

Fresh sterile MRS agar, incubated for 24 h and another round of plasmid extraction performed to ascertain the success or otherwise of curing. The LAB that showed no plasmids were finally used for another round of antibiogram using the antibiotics they were resistant to. The absence of resistance thereafter was interpreted as the initial resistance being plasmid mediated.

Statistical analysis

Univariate analysis was performed to determine resistance to ≥ 2 antibiotics. A multinomial logistic regression using IBM SPSS vs 20 was conducted to determine association of LAB resistance patterns with vegetable sources/types and strains at 95% confidence interval (CI).

RESULTS

Antibiogram of LAB

The entire LAB were multi drug resistant, while 97.0% were resistant to cloxacillin, ceftriaxone each and 94.0% resistant to vancomycin. All the LAB except one (uncultured Solibacillus) was sensitive to imipenem. Rifampicin was the next in terms of activity against the LAB after imipenem as 91.4% of the LAB were sensitive to this antibiotic. This was followed by clindamycin (Table 3).

Plasmid analysis

Seventy-four percent (74.0%) of the selected LAB isolates possessed less than 2 kb plasmid while 83.0% carried 23 kb. Forty-two percent (42.0%) carried at least two copies each. LAB from cucumber carried most of the plasmid (38.0%), followed by cabbage (32.0%), lettuce (27.0%) and carrot (3.0%). The pattern of Antibiogram remained basically the same as in pre-curing experiment.

PCR detection of resistance genes

The results of PCR showed four different LAB strains carrying 454 bp vancomycin resistance (*van*X) genes. These LAB isolated mostly from Oke-Odo are as follows, uncultured Solibacillus clone RBL- 135, *L. plantarum* YML 007, *L. plantarum* TCP 008, *W. cibaria* PON 10339. *Weissella confusa* SJL 602 from lettuce amplified 325 bp resistant gene for beta lactam (*bla*Z).

Three other LAB, *L. fermentum* LG1, *L. brevis* BB7 (both from carrot) and *L. fermentum* PBCC11 from lettuce amplified this gene (*bla*Z) but corresponding to a larger size of 500 bp. None of the genes encoding tetracycline ribosomal protection proteins (RPPs) (*tet*S, *tet*W, *tet*M), efflux (*tet*K, *tet*L), aminoglycosides resistance *aac*(6^1)-*aph* (2^1) and also integrase enzyme *intl* 1, 2, 3 was

Table 4. Primers, genes and PCR conditions for antibiotics resistance in LAB.

Target gene	Primer	Sequence (5^1- 3^1)	Size (bp)	Annealing temperature (^oC)	PCR conditions	Reference
tet (M)	tetM- F tetM- R	GTGGACAAAGGTACAACGAG CGGTAAAGTTCGTCACACAC	406	58	94°C for 5 min, 30 cycles 94°C for 1 min, annealing temp for 1 min, 72°C for 2 min, 72°C for 10 min.	Ng et al., 2001
tet (S)	tetS- F tetS- R	ATCAAGATATTAAGGAC TTCTCTATGTGGTAATC	573	50		Gevers et al., 2003
tet (W)	tetW- F tetW- R	GAGAGCCTGCTATATGCCAGC GGGCGTATCCACAATGTTAAC	168	60		Aminov et al., 2001
tet (K)	tetK- F tetK- R	TTAGGTGAAGGGTTAGGTCC GCAAACTCATTCCAGAAGCA	697	58		Aarestrup et al., 2000
tet (L)	tetL- F tetL- R	CATTTGGTCTTATTGGATCG ATTACACTTCCGATTTCGG	456	56		Aarestrup et al., 2000
aac(6')-*aph*(2')	aac- F aac- R	CCAAGAGCAATAAGGGCATA CACTATCATAACCACTACCG	230	58	95°C for 30 s, 58°C 45 s, 72°C 2 min; 30×	Rojo-Bezares et al., 2006
bla(Z)	blaZ- F blaZ- R	TACTTCAACACCTGCTGCTTTCG CATTACACTCTTGGCGGTTTCAC	325	61	95°C for 30 s, 61°C 45 s, 72°C 2 min; 30×	Liu et al., 2009
van(X)	vanX- F vanX- R	TCGCGGTAGTCCCACCATTCGTT AAATCATCGTTGACCTGCGTTAT	454	60	95°C for 30 s, 60°C 45 s, 72°C 2 min; 30×	Liu et al., 2009
Intl-1,2,3	hep 35 hep 36	TGCGGGTCAAGGATTTGGATTT CAGCACATGCGTATAAAT	491	54	94°C for 2 min, 35 cycles of 94°C for 30s, 54°C for 45 s, 72°C for 45s, 72°C for 7 min	White et al., 2000

tet- Tetracycline resistant gene, aac (61) - aph (21) - aminoglycoside acetyltransferase and phosphotransferase encoding gene, bla- beta lactamase gene, van- vancomycin resistance gene, intl- integrase encoding gene.

amplified by any of the LAB.

Statistical analysis

The vegetable sources/types, LAB strains have no statistical significance with resistance patterns ($p>0.05$).

DISCUSSION

The identities of resident LAB in cabbage, carrot, cucumber and lettuce employed in this study were confirmed by partial sequencing of their 16S rRNA genes. This according to European Food Safety Authority is the first approach in the study concerning AR gene/safety of foodborne bacteria (EFSA, 2012). The occurrence of *Lactobacillus* spp. in largest number is in tandem with various reports (Tamang et al., 2005; Gomes et al., 2010; Gad et al., 2014). Salad vegetables are served and eaten raw in Nigeria and indeed many other climes as such, any AR borne on conjugative mobile genetic elements poses a great danger to the populace. Our investigation of linkage of mobile genetic elements, plasmids and integrons to AR in LAB is the first in the locally grown salad vegetables. The Antibiogram of LAB is still interpreted with a great deal of inconsistencies (Franz et al., 2005; Hummel et al., 2007) as the phenomenon of break-point/cut off values are yet to be duly validated in many LAB strains. This is partly due to the fact that LAB AR issues emerged barely 2 decades ago as they have ever been generally regarded as safe and the growth medium for LAB (MRS) renders the activities of test antibiotics sub optimal. In this study, the LSM, as demonstrated by Klare et al. (2007), was employed and susceptibilities were interpreted based on CLSI (2007) guidelines. Majority of the LAB were phenotypically multi drug resistant but this did not correlate with their possession of respective AR genes. For instance, *tet* gene for tetracycline frequently reported in food borne LAB was not detected in this study. The tetracycline ribosomal protection proteins (RPPs) and efflux pump were not detected. This may be associated with the phenomenon of 'silent' gene or inducible AR genes often reported in LAB. For instance, Hummel et al. (2007) reported some LAB strains possessing silent chloramphenicol acetyltransferase (*cat*) gene under inducing and non-inducing conditions. In phenotypic terms, most of the LAB were susceptible to erythromycin. The resistance of LAB to glycopeptides, vancomycin is infrequently reported especially in *Weissella* spp. unlike the findings in this study where the resistance gene (*van*X) was detected in *W. cibaria* PON 10339 alongside other LAB strains. The resistance to this antibiotic is

reported to be intrinsic in *Lactobacillus* spp. All the LAB carrying *van*(X) gene were phenotypically resistant to vancomycin. This innate resistance of LAB to vancomycin has been demonstrated to be due to replacement of D-ala- D- ala precursor of muramyl pentapeptide in peptidoglycan in LAB with D-lact or D-ser (Nelson, 1999; Mathur and Singh, 2005; Ammor et al., 2007). It must be mentioned here that about 9 other vancomycin resistance genes other than *van*(X) have been reported to date and *van*(X) gene is uncommonly detected in LAB in general and *Weissella* spp. in particular. All the vancomycin genes are not usually borne on mobile genetic elements (Klein et al., 2000). Uncultured Solibacillus clone RBL-135 was resistant to all tested antibiotics in this study including imipenem but was greatly inhibited by ofloxacin (data not shown).

The resistances to β-lactams (*bla*Z) antibiotics were pronounced in this study as 97.0% of the LAB were phenotypically resistant to cloxacillin while more than 90.0% were resistant to other β-lactams including cephalosporins. This indicates intrinsic or natural factors. The *bla*(Z) gene detected in *W. confusa* SJL 602 is also uncommonly reported in this LAB. In fact, this search in literature for this gene in this LAB yielded no positive reports as most of the carriers were *Lactobacillus* spp. This gene was amplified in 3 other LAB (*L. fermentum* LG1, *L. brevis* BB7 both from carrot and *L. fermentum* PBCC11 from lettuce) in this study corresponding to unexpected size of 500 bp. This may be due to non-specific binding or mutation. DNA sequencing or DNA-DNA hybridization study should be done to confirm the identity of this gene.

Majority of the LAB possessed plasmids and in fact all the detected LAB carrying AR genes did. The presence of this mobile genetic element is in line with the work of Olukoya et al. (1993) where 80.0% of the LAB from Nigerian fermented foods were carriers of plasmids some up to five with size ranging from 1.8 to 45.0 kb. In this study, the size range was from less than 1.0 to 23.1 kb. Seventy-four percent (74.0%) of the LAB carried plasmids while 42.0 and 39.0% carried 2 and 3 copies, respectively. In this study also, the method of Klaehammer (1984) was modified to isolate the plasmid; briefly, the combination of enzymes, lysozyme (5 mg/ml) and mutanolysin (10 U/ml) was employed and the incubation extended to 30 min at 37°C. The authors hope to investigate the types of plasmid (in terms of their biotechnological/functional qualities) borne by these LAB in future.

The non-linkage of plasmid to resistances seen in this study prompted us to assay for integrase enzyme carrying gene cassettes with resistance to up to 10 antibiotics at the same time (Clementi and Aquilanti, 2011). None of the LAB in this study possessed integrons.

The main gap in this study was the non-inclusion of assay for conjugative transposons Tn 916-1545 and insertion sequences (IS) elements which have also been implicated in HGT of AR genes in food borne LAB (Clewell et al., 1995; Churchward, 2002; Hummel et al., 2007; Devirgiliis et al., 2009).

In conclusion, the identified LAB in the study cabbage, carrot, cucumber and lettuce grown locally were phenotypically multidrug resistant, most possessing plasmid DNA which is not linked to the AR based on post curing outcomes. A few were detected to possess vancomycin, *van*(X) and β-lactams, *bla*(Z) resistance genes and none possessed the multi-drug resistant carriers, integrase enzyme. The LAB therefore can be said to be safe in terms of their ability to transfer AR genes through plasmid and integrons based on this study. There is also the need to scale up this study to other regions of the country and initiate biosafety surveillance of LAB in salad vegetables.

CONFLICT OF INTEREST

The authors hereby declare that there is no conflict of interest whatsoever.

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Enhanced production of alkaline protease by *Aspergillus niger* DEF 1 isolated from dairy form effluent and determination of its fibrinolytic ability

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Proteases constitute most important enzymes owing to their wide variety of functions and have immense applications in various industries *viz*., medical, pharmaceutical, biotechnology, leather, detergent, and food industries. Despite of their wide spread occurrence in various sources, microorganisms present remarkable potential for proteolytic enzymes production due to their extensive biochemical diversity and susceptibility to genetic manipulation. The present study was aimed at isolating alkaline protease producing fungal members from dairy form effluents, designing the process variables for maximizing the protease production and determining the fibrinolytic potential of the partially purified alkaline protease. To achieve the specified objectives, the dairy form effluent was processed for the isolation of proteolytic fungi using suitable microbiological medium. All the fungal isolates were screened for their protease producing ability and the isolate showing highest alkaline protease production was selected for further studies. Optimization of different fermentative variables like carbon, nitrogen sources, pH, temperature and incubation period were carried out to enhance enzyme production. Ammonium sulphate fractionation was employed to partially purify the enzyme following which its fibrinolytic potential was determined. Based on morphological and microscopic studies, the selected fungal isolate was identified as *Aspergillus niger*. Optimization studies using OVAT (one variable at a time) method revealed an enhanced protease production in the presence of fructose as additional carbon source and ammonium sulphate as nitrogen source. The optimum incubation period, temperature and pH for enzyme production by the selected fungal isolate was found to be 92 h, 50°C and 10, respectively. The partially purified alkaline protease was efficient in the removal of blood stains emphasizing its fibrinolytic ability. An alkaline protease producing Fungal sp. was screened and isolated from dairy form effluent and it was found to be efficient in the removal of blood stains proving its fibrinolytic potential. Enzymes produced from microorganisms that can survive under extremes of pH could be particularly useful for commercial applications under high alkaline conditions.

Key words: Alkaline protease, dairy form effluent, optimization, *Aspergillus niger*, fibrinolytic potential.

INTRODUCTION

Proteases or proteinases are enzymes which carryout proteolysis by the hydrolysis of their peptide bonds (Anshu and Khare, 2007; Kalpana Devi et al., 2008). Among the industrial enzymes, proteases are one of the

most important groups contributing to the largest sales after carbohydrases and they account for a quarter of the total global enzyme production (Kalaiarasi and Sunitha, 2009). They are ubiquitous and widely distributed in plants, animals and microbes with microbes as major sources among all (Amrita Raj et al., 2012). Fungi harbor a variety of proteolytic enzymes and are important sources compared to bacteria because of their ability to grow at extremes of temperature, pH and as well use a wide variety of substrates as nutrients. Their importance is mainly due to their wide spread applications in various industries *viz*., detergent industry, leather processing, food industries, textile industry, pharmaceutical etc. (Deng et al., 2010; Jellouli et al., 2009). Considering the importance of proteases it is necessary to screen and isolate novel protease producing microbes from different sources. It is ideal that proteases isolated from microbes should maintain higher activities over a broad range of temperature, pH etc. to be used in various industrial applications (Johnvesly and Naik, 2001). Thermo stable enzymes, in addition to their temperature resistance have additional advantages including: Increasing the diffusion and mass transfer rate as the viscosity decreases at high temperatures, enhancement of the reaction rate constant, and increasing the solubility of the hydrophobic substrates. Besides these, high temperatures also lower the risk of contamination by pathogenic microbes (Al-Qodah et al., 2013). Enzymes from microorganisms that can survive under extreme pH may be useful for commercial applications under high alkaline reaction conditions, e.g., in the production of detergents (Denizci et al., 2004). Based on their optimal pH, proteases are categorized into neutral, acidic and alkaline proteases and among them alkaline proteases have major application in the detergent industry as the pH of the detergents usually lies in the range of 9 to 12. Thus, search for new proteases from different sources with novel properties is desirable in order to produce proteases of high performance (Al-Qodah et al., 2013).

In general the effluents of meat, dairy form and poultry industries serve as prime sources of proteolytic microbes owing to their protein rich content (Vishwanatha et al., 2010). In the present study, proteolytic fungi were screened and isolated from dairy form effluent; process variables were optimized for maximizing alkaline protease production and the ability of selected isolate was tested for its fibrinolytic activity.

MATERIALS AND METHODS

Sample collection and isolation of proteolytic fungi

Dairy form effluent was aseptically collected from the Creamline Dairy Products Ltd., Epuru, using sterile spatula and the effluent was carefully packed in a zip lock cover that was pre-sterilized. The collected sample was processed within an hour or stored in the refrigerator if not immediately used. Alkaline protease producing fungal strains were isolated by subjecting the effluent to 10 fold serial dilution and by inoculating the serially diluted sample on modified seed medium (Reese et al., 1950) of pH 9.0. Table 1 shows the composition of modified seed medium. The plates were then incubated at 40°C for a period of 5 days. The alkaline protease producing fungal isolates were identified based on zone of casein hydrolysis around the colonies.

Identification of the fungal isolates

The alkaline protease producing fungi were identified based on morphological characteristics and microscopic observation of fungal spores using lactophenol cotton blue staining. For morphological characterization, the fungal isolates were cultivated on czepakdox agar medium. The shape, size, arrangement and development of conidiophores, phialides and conidiospores were studied using the taxonomic tools of Hoog et al. (2000).

Protease assay

The proteolytic activity of the isolated fungal species was estimated by the method of Udandi Boominadhan et al. (2009). The enzyme activity was estimated in a reaction mixture containing 2.0 ml of 0.5% casein solution in 0.1M Carbonate –Bicarbonate buffer (pH 9.5) and 1 ml crude enzyme extract in a final volume of 3.0 ml. The reaction mixture was incubated at 40°C for a period of 5 min followed by termination of the reaction by the addition of 3 ml of ice-cold 10% TCA (Tri Chloro Acetic acid). After 1 h incubation at room temperature, the precipitate so formed was collected by filtering the contents using Whattman no. 1 filter paper. The tyrosine released by the action of crude enzyme extract on protein was estimated by adding 5 ml of 0.4 M Sodium carbonate and 0.5 ml of Folin phenol reagent to 1 ml of filtrate. This also aid in color development. The mixture was then vortexed immediately and incubated for 20 min at room temperature. The tyrosine released was estimated by measuring OD at 660 nm against the enzyme blank using UV-VIS spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µg of tyrosine per mL per min under standard assay conditions.

Optimization of process parameters by one variable at a time (OVAT) method

The alkaline protease production by the fungal isolate (DEF 1) yielding highest protease was further optimized using OVAT method. The production medium (Nehra et al., 2002) consists of (g L^{-1}) Glucose (10), Casein (5), Yeast extract (5), Ampicillin (0.05), K_2HPO_4 (1), $MgSO_4.7H_2O$ (0.2), Na_2CO_3 (10) and pH (8.5). For optimization, the production medium was supplemented with different additional carbon sources 1% (w/v) (glucose, fructose, sucrose and maltose) and 1% (w/v) nitrogen sources (peptone, yeast extract, beef extract, ammonium sulphate, ammonium chloride and sodium nitrate). The effect of various physical parameters such as pH (5, 6, 7, 8, 9, 10, 11 and 12), temperature (25, 30, 35, 40, 45 and 50°C) and incubation time (48, 72, 96 and

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Table 1. Composition of modified seed medium.

Media component	Quantity (g/L)
Glucose	2.5
Casein	5
Ampicillin	0.05
pH	9

Fungal members were screened by inoculating dairy form effluent on modified seed medium and incubating the plates at 40°C for a period of 5 days.

Figure 1. Creamline dairy products Ltd. Epuru, Hanuman Junction, Krishna district.

120 h) were also optimized by OVAT method. Submerged Fermentation (SmF) studies were carried out in 250 mL Erlenmeyer flasks containing 45 mL production medium inoculated with 3% inoculums and incubated for 4 days in an orbital shaker (150 rpm) at 40°C. Fungal biomass was separated by centrifugation at 10,000 rpm for 10 min and the filtrate was considered as a crude enzymatic extract and stored at -20°C for further use.

Partial purification of protease

The crude protease extract obtained by culturing *A. niger* DEF 1 in the optimized protease production medium was subjected to ammonium sulphate fractionation (0-20, 20-40, 40-60 and 60-80%). The precipitates so obtained by centrifugation were dissolved in 0.1 M phosphate buffer (pH 7) and dialyzed against same buffer for overnight at 4°C. The samples were then tested for protease activity using protease assay.

Determination of fibrinolytic activity of partially purified protease

Fibrynolytic potential of partially purified protease was determined by incubating 3 pieces of cotton fabric impregnated with blood. The dried blood stains were fixed with 2% (v/v) formaldehyde for 30 min and the excess formaldehyde is removed by rinsing with water (Adinarayana, 2003). The cotton fabrics were then allowed to dry. After drying, the cotton fabrics were incubated separately with 2 ml of partially purified protease along with detergent, 2 ml of distilled water with detergent and 2 ml of distilled water at 40°C for 1 h. The cotton fabrics were then rinsed with water and checked for the removal of blood stains following drying.

Statistical analysis

Effect of each variable was studied in triplicates and the results were graphically presented as the mean ± standard deviation.

RESULTS

Isolation and screening of proteolytic fungi

A total of 7 different alkaline protease producing fungal sp. were screened and isolated from dairy form effluents collected from Creamline Dairy Products Ltd. Epuru. Figure 1 shows the Creamline Dairy Products Ltd situated at Epuru, near Hanuman Junction, Krishna District. Figure 2 shows the huge heap of left over milk packets dumped in the dairy form premises. Table 2 shows the fungal sp. isolated from the dairy form effluent. Figure 3 shows the isolated fungal sp. (DEF 1 to DEF 7). The proteolytic potential of the fungal isolates was evident by the formation of clear zones around the colonies on skimmed milk agar medium (Figure 4). Of the isolated fungal sp. DEF 1 exhibited highest enzyme production (94 U/mL) and highest zone of proteolysis (20 mm) compared to other isolates (Tables 3 and 4). So it was selected for further optimization studies.

Identification of the selected fungal isolate

The highest protease producing fungal isolate was identified as *A. niger* based on the morphological characters and microscopic observation of spores using lactophenol cotton blue. The morphological and microscopic characteristics of the selected isolate were given in Table 5.

Optimization of process parameters using OVAT method

Effect of incubation period

As the growth phase of the fungus lasts for about 7 to 10 days and the log phase lies in between 3 and 5 days, it is our interest to determine the time of incubation at which lipase production is maximum by *A. niger* DEF 1. So the protease production as factor of incubation time was studied using shake flasks up to 144 h. From the data represented in Figure 5a, it was evident that maximum enzyme yield was obtained after 96 h of incubation period.

Figure 2. A huge heap of milk packets thrown in the dairy form premises.

Table 2. List of fungal members isolated from dairy form effluent.

S/N	Fungal isolate
DEF 1	*Aspergillus* sp.
DEF 2	*Rhizopus* sp.
DEF 3	*Mucor* sp.
DEF 4	*Penicillium* sp.
DEF 5	*Aspergillus* sp.
DEF 6	*Aspergillus* sp.
DEF 7	*Fusarium* sp.

*DEF = Dairy form effluent fungi. 7 different fungal isolates were screened and isolated from dairy form effluent inoculated on modified seed medium.

Effect of incubation temperature

Temperature of the medium plays an important role on metabolic activities of living organisms, in the present case on protease production. As the low temperature shuts or slows down the metabolic activity and high temperature inhibits or deactivates the enzymes or hormones, we studied the effect of temperature on enzyme production by incubating the fungus at different temperatures from 25 to 70°C and from the results obtained it was found that the enzyme production was maximum at 50°C (Figure 5b).

Effect of the medium pH

pH of the medium is an important parameter that affects the growth and metabolism of the organism. Most of the organisms prefer pH from range 5 to 8 for their optimal growth. However, few prefer acidic pH and few others prefer basic pH based on their secretory products. Hence in the present study, the effect of pH on lipase production was studied by culturing the fungus in the protease production medium for 96 h with a pH range 5 to 11 and from the results obtained it was found that optimum pH for protease production was pH 10 (Figure 5c).

Effect of additional carbon source

Carbohydrates like glucose, fructose and sucrose are the most preferred carbon sources to any organisms as they can be readily used up. To check whether carbon source in the form of carbohydrates affect the lipase production, various sugars *viz*., glucose, fructose, maltose and sucrose at a concentration of 1% were added as additional carbon sources to protease production medium. The data obtained (Figure 5d) indicates that fructose enhanced the enzyme production compared to other carbon sources.

Effect of nitrogen sources

The growth of the microbes and enzyme production requires nitrogen sources. Most of the microorganisms require fixed nitrogen sources to synthesize proteins, nucleic acids and other cellular components. The nitrogen sources may be provided as pre-digested polypeptides, as bulk proteins or as nitrate or ammonium salts depending upon the enzyme capabilities of the microorganisms. Hence in the present study, the effect of nitrogen sources on protease production by *A. niger* DEF 1 was studied by incubating the organism in protease production medium with various organic nitrogen sources (1%) *viz*., peptone, yeast extract, beef extract etc. and

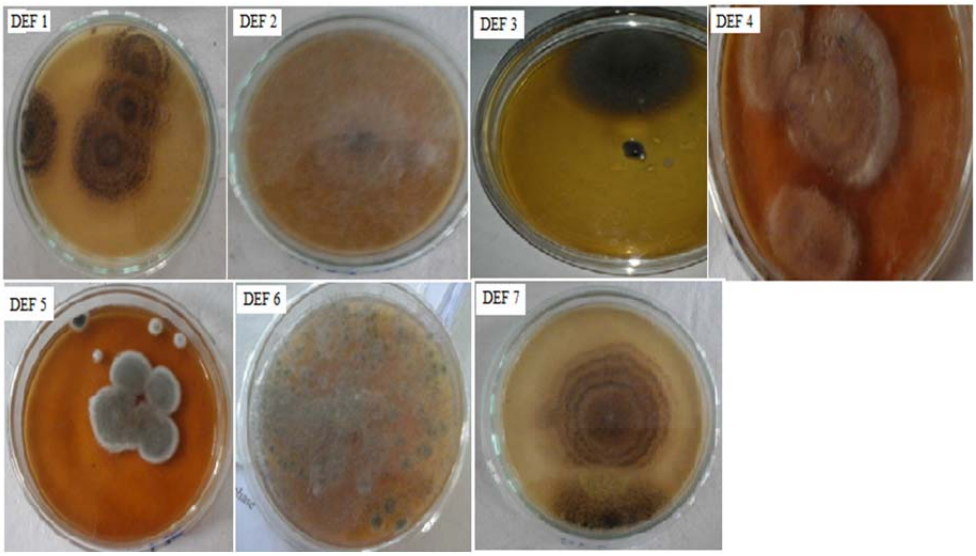

Figure 3. Fungal sp. isolated from dairy form effluent.

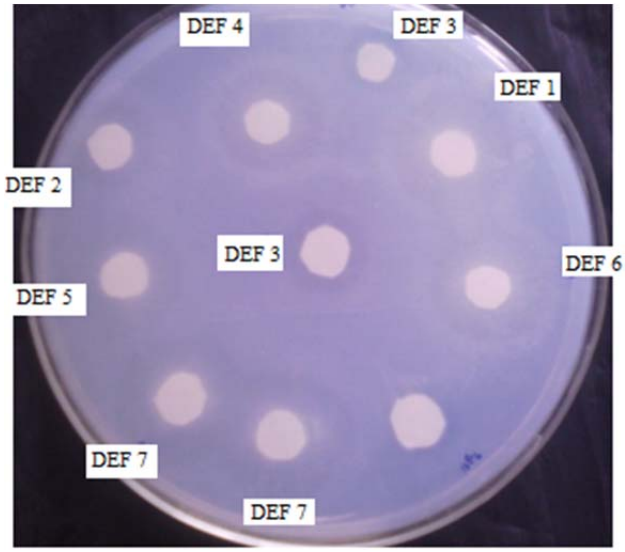

Figure 4. Pertriplate showing zone of proteolysis by the fungal species isolated from dairy form effluent.

with various inorganic nitrogen sources (1%) *viz.,* ammonium sulphate, ammonium chloride and sodium nitrate. From the data obtained (Figure 5e), it was evident that the best nitrogen source for protease production by *A. niger* DEF 1 was found to be Ammonium sulphate.

Partial purification of protease

The highest protease activity was found with 40 to 60% precipitate and hence it was used for further studies.

Fibrinolytic potential of the protease

The fibrinolytic potential of partially purified protease was found in terms of degree of blood removal from the cotton fabric and from the results obtained, blood removal was found in the order of partially purified protease with detergent > distilled water along with detergent > distilled water (Figure 6).

DISCUSSION

Proteases, having large number of commercial applications account for more than 60% of the total enzyme market. They are the oldest enzymes known to mankind and catalyze the hydrolysis of proteins (Raju et al., 1994). They are widely distributed in the nature and are very much essential for cell growth and differentiation (Vadlamani and Parcha, 2011). Despite of their wide spread occurrence in a variety of sources, microbes have proven to be common sources due to their biochemical and physiological properties, ease of genetic manipulation and facile culture conditions. Many researchers are continuously engaged in screening for potential protease producers as these enzymes constitute 2/3rd of the total enzymes used by various industries and this demand is supposed to increase as the years pass by. In recent years also there were several reports on screening and isolation of protease producers (Chandrasekaran et al., 2015; Sharma et al., 2015; Sonia sethi et al., 2015; Rupali et al., 2015; Tiwari et al., 2015) indicating the importance of these industrial enzymes. Hence the present study was undertaken to isolate potential alkaline protease producing fungi from dairy form effluents as these effluents provide alkaline environment for the

Table 3. Fungal isolates and their corresponding protease activity in units/mL.

S/N	Fungal Isolates	Protease activity (Units/mL)
DEF 1	*Aspergillus* sp.	94
DEF 2	*Rhizopus* sp.	20
DEF 3	*Mucor* sp.	15
DEF 4	*Penicillium* sp.	36
DEF 5	*Aspergillus* sp.	27
DEF 6	*Aspergillus* sp.	82
DEF 7	*Fusarium* sp.	86

For the estimation of protease activity, the fungal spores were inoculated in 45 mL of production medium (pH 8.5) and the flasks were incubated at 40°C for a period of 72 h. The fungal biomass was then separated by centrifugation at 10,000 rpm for 10 min and the filtrate was considered as a crude enzymatic extract

Table 4. Fungal isolates and their corresponding protease activity in mm.

S/N	Fungal Isolate	Protease activity (mm)
DEF 1	*Aspergillus* sp.	20
DEF 2	*Rhizopus* sp.	7
DEF 3	*Mucor* sp.	5
DEF 4	*Penicillium* sp.	15
DEF 5	*Aspergillus* sp.	10
DEF 6	*Aspergillus* sp.	17
DEF 7	*Fusarium* sp.	19

The qualitative test for protease activity was done by placing the filter paper discs immersed in crude enzyme extract on skim milk agar medium following incubation of the plates at 40°C for a period of 24 h. The zone of casein hydrolysis around the disc was identified as protease activity and was measured in mm.

growth of alkaline protease producers.

In the present study, effluent was collected from Creamline Dairy Products Ltd. Epuru, Krishna District. 7 different alkaline protease producing fungal members were screened and isolated from the effluent samples using skim milk agar medium. The fungal isolate producing highest protease was found to be *A. niger* DEF 1. Alkaline protease production by *A. niger* was also reported by earlier workers (Kalpana Devi et al., 2008; Coral et al., 2003; Abidi et al., 2014) from different sources. For the first time, we are reporting an alkaline protease producing *A. niger* from dairy form effluent. The highest protease production was found in the presence of fructose as additional carbon source compared to glucose which could be due to catabolite repression by high glucose available in the production medium. Mukhtar and Ikram-Ul-Haq (2009) also reported the same observation with acid protease from *A. niger*. Higher protease yields were reported by several workers in the presence of a variety of carbon sources such as glucose, 1% wheat bran, 1% starch and corn steep liquor etc (Malathi and Chakraborty, 1991; Kalpana Devi et al., 2008; Vaishali and Jain, 2012; Palanivel et al., 2013).

Protease production is known to be influenced by the presence of nitrogen sources in the production medium and different organisms prefer different nitrogen sources for their growth and enzyme production (Singh et al., 2011). Earlier studies revealed significant protease production in the presence of organic nitrogen sources (Malathi and Chakraborty, 1991; Mukhtar and Ikram-Ul-Haq, 2009; Mostafa El-Sayed et al., 2012; Palanivel et al., 2013; Sonia sethi et al., 2015) and reduced growth and enzyme production in the presence of inorganic nitrogen sources such as ammonium compounds (Sehar and Hameed, 2011). In contrary to their findings, the present study reported higher protease production in presence of ammonium sulphate. Similar finding was also reported by Kalpana Devi et al. (2008). There were so many reports for higher protease production in the presence of inorganic nitrogen sources (Rajkumar et al.,

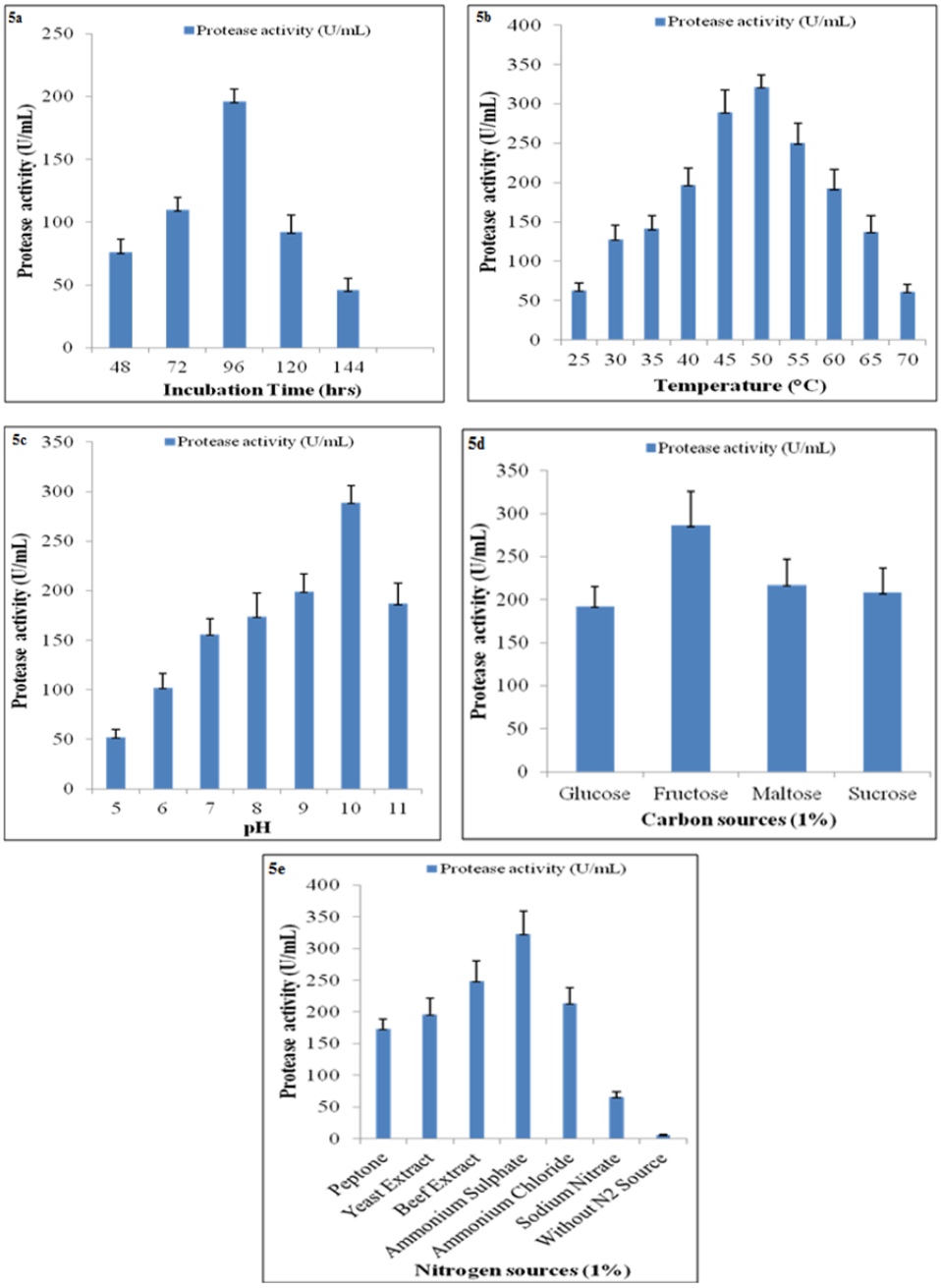

Figure 5. Optimization of process variables for enhanced alkaline protease production by *A. niger* DEF 1. (a) Effect of incubation period on protease production - Spore suspension was inoculated into production medium and incubated for different time periods viz., 48, 72, 96, 120 and 144 at 40°C followed by enzyme estimation. (b) Effect of incubation temperature on protease production - Spore suspension was inoculated into production medium and incubated at different temperatures from 25 to 70°C for 96 h at 150 rpm and enzyme activity was estimated. (c) Optimization of pH for protease production - Spore suspension was inoculated into production medium with varying pH ranging from 5 to 11 and incubated at 50°C for 96 h at 150 rpm and enzyme activity was estimated. (d) Effect of additional carbon sources on protease production - Spore suspension was inoculated into production medium of pH 10 with 1% of various sugars as additional carbon sources viz., glucose, fructose, maltose and sucrose etc. and incubated at 50°C for 96 h and enzyme activity was estimated. (5) Effect of nitrogen sources on protease production - Spore suspension was inoculated into production medium of pH 10 containing 1% fructose as additional carbon source with 1% each of various organic and inorganic nitrogen sources viz., Peptone, Yeast extract, Beef extract, ammonium sulphate, ammonium chloride and sodium nitrate etc. incubated at 50 °C for 96 h and enzyme activity was estimated. The production medium without nitrogen was used as control to show the importance of nitrogen for cell growth and enzyme production.

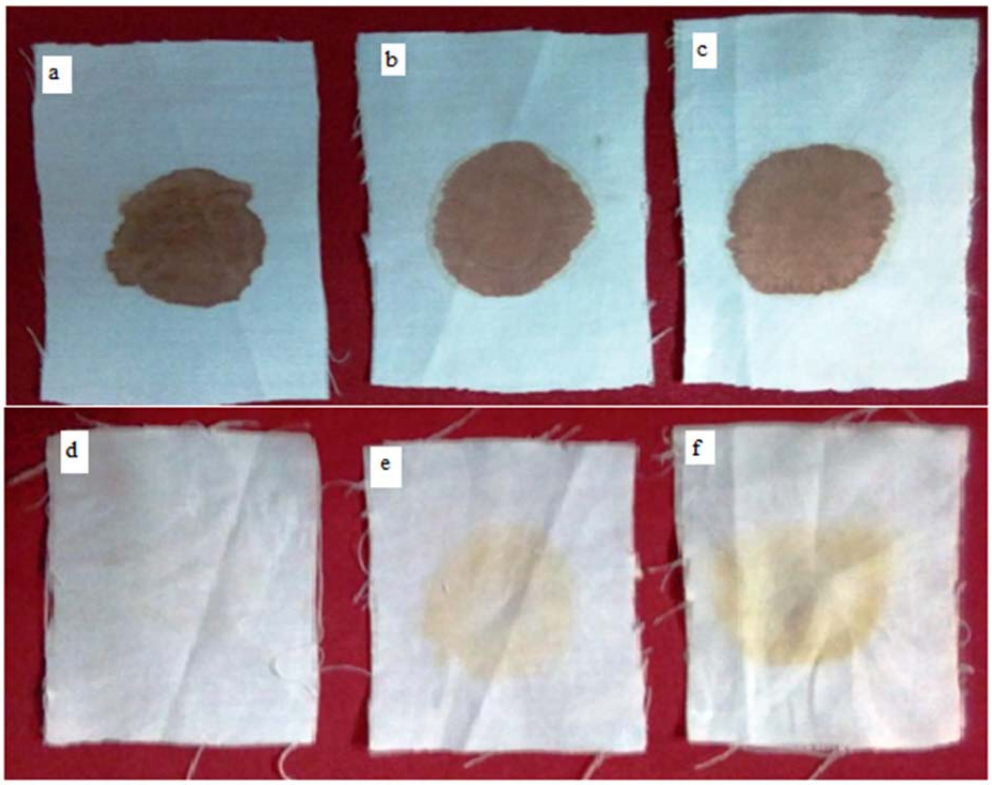

Figure 6. Determination of fibrynolytic potential of partially purified alkaline protease from *A. niger* DEF 1. a, b and c denotes cotton fabrics stained with blood sample; d, e and f represents stained cotton fabrics washed separately with partially purified protease along with detergent, distilled water with detergent and distilled water respectively.

Table 5. Morphological and Microscopic characteristics of *A. niger* DEF 1 isolated from dairy form effluent.

Characteristics of *Aspergillus niger* DEF 1	
Morphological	
Surface colour	Dark brown to black spores
Reverse side of the colony	Without colour
Margins	Entire
Elevations	Umbonate
Growth	Rapid
Microscopic	
Hyphae	Branched septate
Conidiophore	
Length	300 µm
Diameter	8 µm
Vesicle	Globose
Conidia	50 µm
Length	Blackish brown
Diameter	3 µm
Ornamentation	Spiny exine
Phialides	Biseriate
Cleistothecia	Present

The morphological characters of the fungal isolate, DEF1 (*A. niger*) were determined by growing the fungus on Czepekdox agar medium and the microscopic characteristics were determined by spore staining using lacto phenol cotton blue.

2010; Vaishali and Jain, 2012).

pH of the fermentation medium is one of the most important factor affecting enzyme production as microbial cells have no mechanism to adjust their internal pH and hence alteration in optimum pH range results in poor growth of microbes and hence poor enzyme production (Bhattacharya et al., 2011). In the present study a pH of 10 was found to be optimum for protease production. Alkaline proteases were found to have wide range of applications in detergent industry. Alkaline protease production was also reported by Kalpana Devi et al. (2008), Oyeleke et al. (2010), Vaishali and Jain (2012) and Palanivel et al. (2013) from different *Aspergillus* sp. Sonia and Saksham (2015) reported an optimum pH of 9.0 for the protease produced by *Penicillium chrysogenum* isolated from the soil. An optimum pH of 8.0 and 7.0 was reported by Chandrasekaran et al. (2015) for proteases produced by *Aspergillus flavus* and *A. niger* respectively isolated from paddy soil. Radha et al. (2011) and Mukhtar and Ikram-Ul-Haq (2009) reported optimum acid protease production by *Aspergillus* sp.

Incubation temperature also plays an important role in enzyme production and growth of microbes can be activated at one temperature and inhibited at another. It also influences the synthesis and secretion of enzyme production by changing the physical properties of the cell membrane (Balaji et al., 2012). In the present study, an optimum protease production was obtained at 50°C. Hence it was a heat stable protease. Ibrahim et al. (2009) reported maximum cell growth at 50°C and enzyme production at 37°C for alkaline protease production by *Bacillus* sp. An optimum incubation temperature of 40°C (Coral et al., 2003) and 45°C (Kalpana Devi et al., 2008) was reported for different *A. niger.* Chandrasekaran et al. (2015) reported an optimum temperature of 30 and 35ºC for the proteases produced by *A. flavus* and *A. niger* respectively isolated from paddy soil. An optimum incubation temperature of 35°C was reported for alkaline protease produced by *P. chrysogenum* isolated from soil samples (Sonia and Saksham, 2015).

Optimization of incubation period is very much essential as organisms show considerable variation in enzyme production at different incubation periods (Kumar et al., 2012). In our study, a maximum protease production was found at 96 h. Similar finding was reported for alkaline protease production from *Aspergillus Versicolor* by Vaishali and Jain (2012). Malathi and Chakraborty (1991) reported an incubation period of 48 h for protease production by *A. flavus*. Enhanced extracellular protease production after 144 h of incubation period was reported by Oyeleke et al. (2010) for both *Aspergillus fumigatus* and *A. flavus* isolated from local rice husk dump sites. Highest alkaline protease production was reported after 168 h of incubation by *P. chrysogenum* (Sonia and Saksham, 2015).

The enzyme was found to be very effective in removing blood stains from fabrics, hence can be applicable in detergent formulations. Similar finding was reported for alkaline protease from *Bacillus* sp. by Mala and Srividya (2010). Palanivel et al. (2013) reported prompt fibrinolytic activity for partially purified alkaline protease from *Aspergillus* strain KH 17 isolated soil samples collected from Eastern Ghats, Kolli hills region. Our study elucidates the use of dairy form effluents as good sources for isolation of alkaline protease producing fungal sp. The high yield of enzyme at alkaline pH (10) also suggests that this organism can find application in detergent and textile industries. The study gains its importance as proteases have innumerous applications in different industries.

Conclusions

The results of the present study clearly demonstrate that dairy form effluents can be used as potential sources for isolating alkaline protease producing fungi. As proteases accounts for more than 60% of the global industrial enzyme market, the present study gains its importance in isolating heat stable, alkaline protease producing fungal isolate. The high yield and activity of the protease at alkaline pH (pH 10) besides its fibrinolytic potential suggests its important role in various industrial applications especially in detergent formulations.

Conflicts of Interests

The authors have not declared any conflict of interests.

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Combined efficacy of thymol and silver nanoparticles against *Staphylococcus aureus*

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Due to the looming spread of resistance to classical antimicrobial agents, innovative therapeutic methods are in dire need to combat the onslaught of resistant bacterial pathogens. This study examines the antimicrobial efficacy of a phytochemical and a metallic nanoparticle against the top Gram positive resistant pathogen. The potential synergy of these two agents was also evaluated. The antibacterial activity of thymol and silver nanoparticles were tested individually using disc diffusion technique. The extent of synergy of their combination was evaluated using the checkerboard assay. Twenty clinical isolates of *Staphylococcus aureus* characterized as methicillin resistant or methicillin sensitive *Staphylococcus aureus* were utilized and the extent of synergism was calculated from fractional inhibitory concentration indices. Thymol exhibited an antistaphylococcal activity regardless of whether the isolates were phenotypically resistant or sensitive to methicillin. Combining thymol with silver nanoparticles resulted in at least additive or synergistic effect for all the examined strains and methicillin resistant strains were inhibited in the combinatorial assays to a greater extent comparative to when silver nanoparticles or thymol were used singly.

Key words: Silver nanoparticles, thymol, *Staphylococcus aureus*, synergy, fractional inhibitory concentration index.

INTRODUCTION

The everlasting battle between humans and infectious diseases causing pathogens continues. Emerging at the front line of challenges to human health is bacterial resistance and its alarming spread. This ongoing rise in resistance is critically threatening the immeasurable medical advancements made possible by antibiotics over the past 70 years (WHO, 2014). Multi-drug resistant-methicillin resistant *Staphylococcus aureus* (MDR-MRSA) is a constantly evolving paradigmatic pathogen. The silently vicious incarnations of *S. aureus* widespread in

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community and hospital environments have posed serious clinical imbroglio (Magiorakos et al., 2012). More than 95% MRSA worldwide do not respond to first line antibiotics and resistance has even evolved to the more recent antimicrobial agents like linezolid, vancomycin, teicoplanin and daptomycin (Kaur and Chate, 2015).

Aggravating the problem of accelerating bacterial resistance to currently approved antibiotics is the lack of investment in antibiotic discovery by the pharmaceutical industry (Spellberg et al., 2007). This situation is so dire that the World Health Organization has recognized multidrug resistant (MDR) bacteria as one of the top three threats to human health (Bassetti et al., 2011). Novel approaches to combating infections caused by these bacteria are sorely needed. An alternative hope resides in medicinal plants, since nature is the only resource to offer an assortment of chemical compounds that can be utilized for new drug discovery. Moreover, phytohemicals are privileged with their lower mammalian toxicity, lesser environmental effects and wider public acceptance (Paranagama et al., 2003). The array of phytochemicals that have been studied for their antimicrobial activity ranges from phenolic compounds, alkaloids, saponins, glucosinates to terpenoids. Research in this field was not halted by the discovery of antibacterially active phytochemicals but extended to their combination with antibiotics that provided the next approach to combat multidrug resistant bacteria (Nostro et al., 2004).

Nanosilver is a versatile antimicrobial agent with established efficacy against a broad range of microorganisms, including bacteria, yeast, fungi, algae and even viruses (Rai et al., 2009). Recently, silver nanoparticles (AgNPs) were considered predominantly attractive for the production of a novel class of antimicrobials opening up a totally new approach to combat a wide assortment of bacterial pathogens (Morones et al., 2005; Shrivastava et al., 2010). In fact, its broad spectrum of activity against morphologically and metabolically different microorganisms, its multifaceted mechanism, as well as, the rare incidence of resistance to elemental silver puts it forth as a potential candidate in the context of the continuing rise in drug resistant strains of bacteria (Panacek et al., 2006; Franci et al., 2015) .

While the approaches to combat antibiotic resistance encompass an array of perspectives from antibiotic stewardship programs, antibiotic alternatives to antivirulence drugs, combination therapy is the norm in treatment of many infections. One of the potential candidates in this respect is the combination between metallic nanoparticles and phytochemicals that may lead to new choices to overcome the onslaught of microbial resistance. Eventually, the aim of the present study was to assess the susceptibility of staphylococcal isolates, characterized as methicillin sensitive *Staphylococcus aureus* (MSSA) or methicillin resistant *S. aureus* (MRSA), to the phytochemical compound "thymol". Furthermore, the synergistic potential of the combination of both thymol and silver nanoparticles was investigated.

MATERIALS AND METHODS

Bacterial strains isolation and identification

Twenty (20) clinical strains of *Staphylococci* from the routine laboratory of Damanhour Main Hospital were enrolled in the present study. The species level confirmation was done using Gram staining, colonial morphology, coagulase positivity and inoculation onto mannitol salt agar (Oxoid). The strains were categorized as MRSA or MSSA based on cefoxitin disc (30 µg) agar screening method (CLSI, 2015). Standard strains of *S. aureus*: *S. aureus* ATCC 43300 and *S. aureus* ATCC 13150 corresponding to methicillin resistant and sensitive strains, respectively, were also included in the current study.

Synthesis and characterization of citrate coated silver nanoparticles

Citrate coated silver nanoparticles were prepared using silver nitrate as the source material and trisodium citrate as the reducing and capping agent. All chemicals used were of analytical grade. 10 ml of 1mM $AgNO_3$ was heated in a water bath and to this solution, 1 ml of 1% trisodium citrate was added gradually and shaking was done till a pale yellow colour was formed. Heating was ceased and the prepared $AgNO_3$ were left to cool at room temperature. The suspension thus obtained was purified by centrifugation at 12000 rpm for 15 min and the supernatant was discarded. A dried powder of the nanosized silver was obtained by freeze-drying. For characterization and assessment of AgNPs antibacterial activity, the freeze dried powder was resuspended in deionized water (Munro et al., 1995).

The shape and nanodimension of the synthesized AgNPs were assessed using transmission electron microscope (Jeol, Tokyo, Japan) by placing drops of the silver nanoparticles solutions on carbon-coated TEM grids. The particle size distribution of the prepared AgNPs was determined by laser light scattering on a Beckman Coulter Particle Size Analyzer (NS submicron particle size analyzer, Japan). Zeta potential was assessed using Zetasizer Nano ZS (Malvern UK). UV-Vis spectroscopy of 10-fold diluted dispersion of AgNPs was recorded using UV-6800 UV/VIS spectrophotometer (Jenway, Germany).

Susceptibility testing of methicillin sensitive and methicillin resistant *S. aureus* isolates

The susceptibility of the MRSA and MSSA isolates to thymol (Sigma Chemical Co., UK) was evaluated using Mueller Hinton Agar (MHA, Oxoid) as described in the diffusion method commonly used for antibiotics (CLSI, 2015). Sterile paper discs (6 mm in diameter) impregnated with 10 µl of either AgNPs (at final content of 10 µg/disc) or thymol solutions were laid on the surface of MHA plates with inoculations of approximately 10^5 CFU/ml of the respective strain. Thymol was prepared as a 1:10 diluted stock in dimethyl sulphoxide (DMSO). Antimicrobial activity was expressed in terms of the inhibition zone (IZ) diameters obtained following incubation at 37°C for 18-24 h.

Test for synergism by disc diffusion method

The synergistic potential of nanosilver and thymol was evaluated using sterile discs impregnated with 5 µl of both thymol and AgNPs

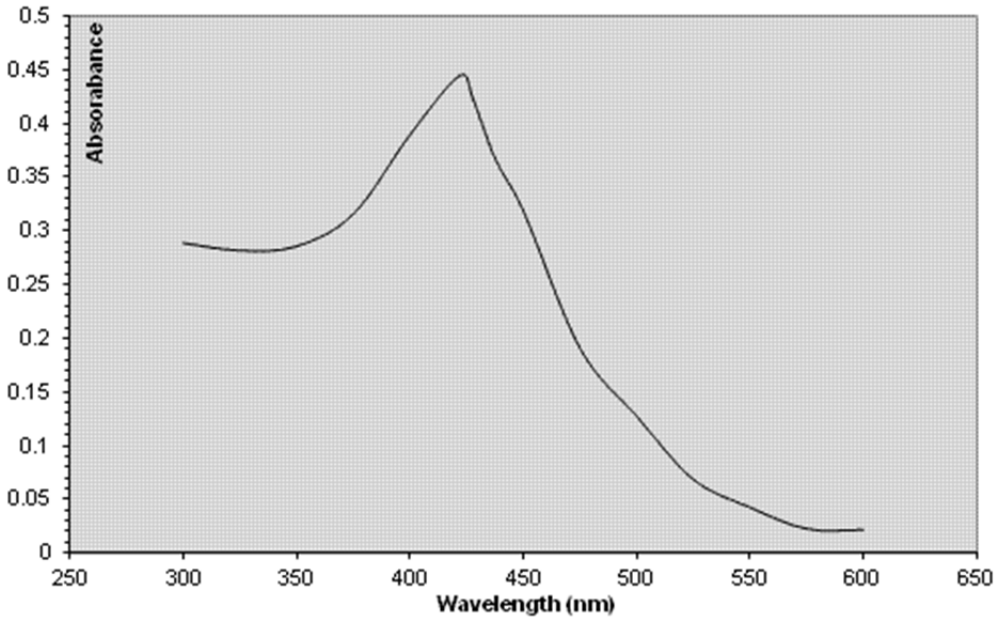

Figure 1. UV-Vis spectrum of citrate coated AgNPs with surface plasmon resonance bend at 422 nm.

and proceeding as aforementioned. The percentage fold increase in inhibition zone was calculated using the formula B-A/A x100 where A and B are the inhibition zones in mm obtained for thymol alone and in combination with AgNPs, respectively (Singh et al., 2013).

Test for synergism of thymol and silver nanoparticles by checker board assay

Minimum inhibitory concentrations (MIC) of thymol and silver nanoparticles were determined individually by the standard microdilution method (CLSI, 2015; Wiegand et al., 2008). Two fold serial dilutions of thymol in DMSO were used comprising 32, 64, 128, 256, 512 and 1024 µg/ml (Hamoud et al., 2014). Simultaneously, the MIC of AgNPs was assessed using gradient concentrations of AgNPs in Mueller Hinton broth (MHB) (5, 10, 20, 40, 80 and 160 µg/ml) (Paredes et al., 2014). A growth control (bacterial inoculum in MHB with 1% DMSO) and a sterility control (MHB with 1% DMSO) were also included in the assay. The MIC was taken as the lowest concentration of the agent that showed no visible turbidity matching with a negative control after incubation at 35°C for 24 h.

The checkerboard dilution method was used to evaluate the *in vitro* synergy of thymol and silver nanoparticles. From the stock solutions of thymol and silver nanoparticles, a two-fold serial dilution to at least double the MIC was distributed in a microtiter plate. The bacterial suspensions were then added to reach inoculums of $5x10^5$ CFU/ml. Thymol dilutions were placed along the abscissa of the microtiter plate in ascending concentrations starting at four dilutions below the MIC and ending at two times the MIC. Meanwhile, AgNPs dilutions were distributed along the ordinates of the plate. The microtiter plate was incubated overnight at 35°C and MIC was read as the lowest concentration of the agent at which no visible growth occurred. The fractional inhibitory concentration index (FICI) was used to evaluate synergy as per the Clinical Laboratory Standards Institute guidelines for broth microdilution (CLSI, 2015). FICI was calculated using the formula FICI=FIC A + FIC B, where FIC A is the MIC of thymol in combination/MIC of thymol alone, and FIC B is the MIC of AgNPs in combination/MIC of AgNPs alone. The combination was considered synergistic when the FICI ≤ 0.5. However, indifference was indicated by an FICI>0.5 and antagonism was depicted by an FICI > 4 (Hsieh et al., 1993; Petersen et al., 2006; Meletiadis et al., 2010).

Statistical analysis

All experiments were done in triplicates. The mean values were calculated and the standard deviation was determined. The IZ diameters of methicillin resistant and methicillin sensitive strains of *S. aureus* were compared using Fisher's exact test.

RESULTS

In the present study, characterization of the prepared AgNPs demonstrated a narrow absorption peak at wavelength of 422 nm (Figure 1). Moreover, spherical, non-aggregated nanoparticles were observed in the TEM micrograph with a diameter range of 21 to 41 nm with an average particle size of 26.9 nm and polydispersity index of 0.73 (Figure 2). A zeta potential of -33.9 mv ± 6.87 reveals high aggregation stability for the prepared AgNPs (Figure 3).

The susceptibility of the MRSA and MSSA isolates to thymol, individually and combined with nanosilver was evaluated using disc diffusion method. The IZ diameter (mm) around the different discs with and without AgNPs against the test strains is shown in Table 1. Silver nanoparticles displayed negligible activity against both resistant and sensitive strains of *S. aureus.* Thymol exhibited an inhibitory effect where relatively larger IZ diameters were observed for MSSA isolates (30-35

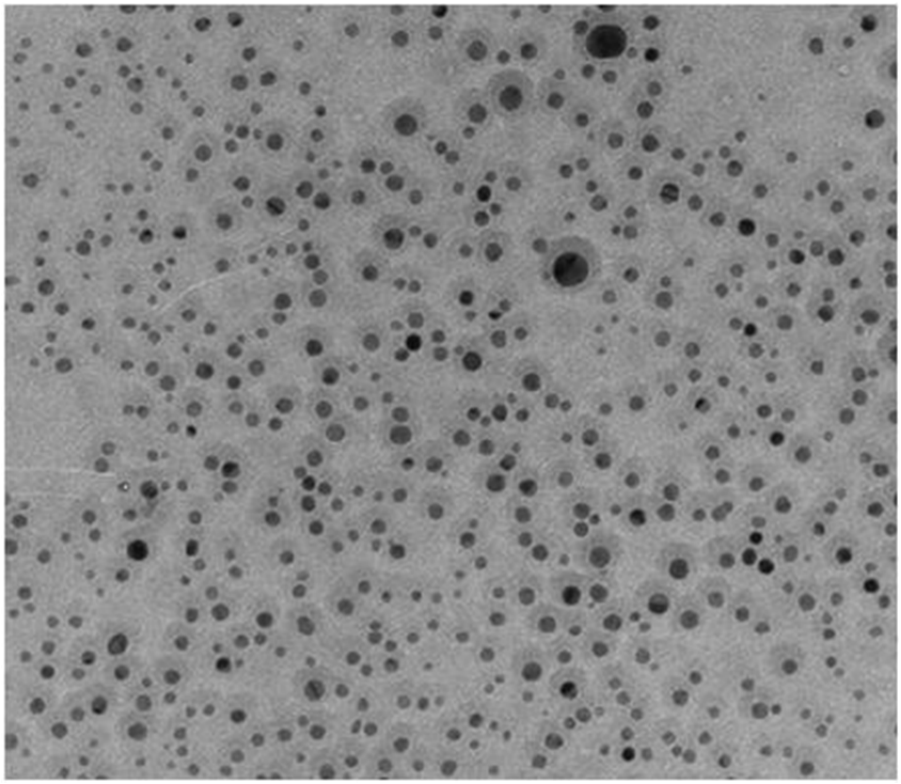

Figure 2. Transmission electron micrograph of prepared AgNPs (Mag. 35000X).

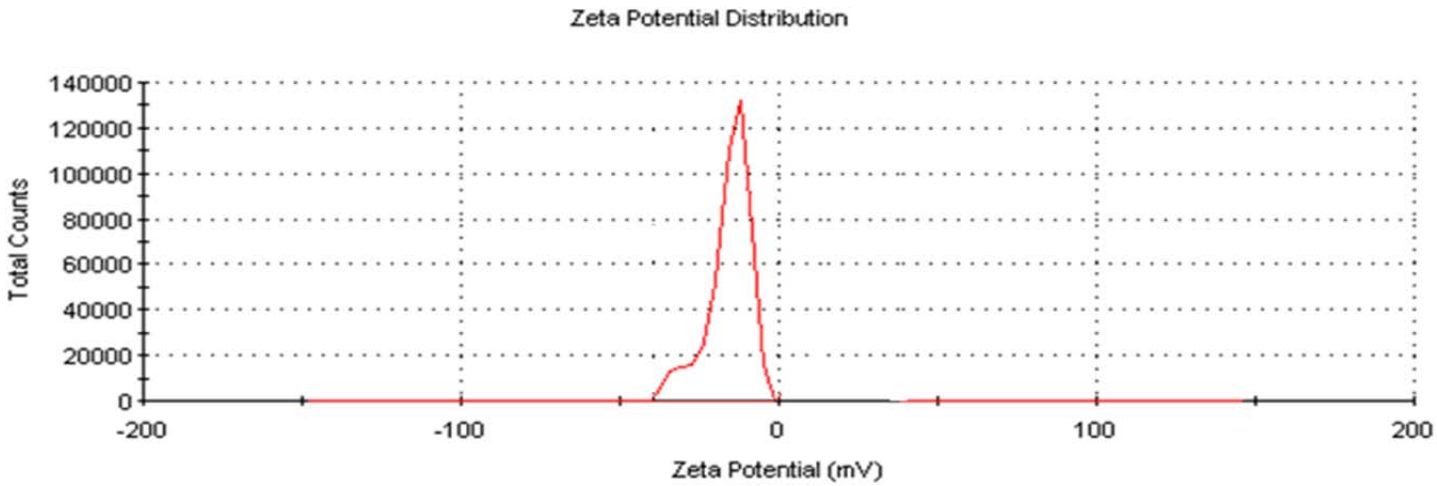

Figure 3. Zeta potential curve of citrate coated AgNPs (Zeta potential = -33.9 mV).

versus 29-34 mm for MRSA). The antibacterial activity of thymol was increased in combination with Ag-NPs, as shown by the fold increase percentage (Table 1).

The minimum inhibitory concentrations of thymol and AgNPs for both MRSA and MSSA were determined by broth microdilution technique. The observed MIC values of thymol and AgNPs for MRSA ranged from 128 to 256 and 40 to 160 µg/mL respectively, while those for MSSA ranged from 128 to 256 and from 20 to 80 µg/mL, respectively (Table 2). The table also shows the FICI results using the checkerboard technique, which demonstrated the mostly synergistic effect of the combination of both thymol and AgNPs, with few indifferent but no antagonistic combinations.

Table 1. Staphylococcal susceptibility to thymol and silver nanoparticles in terms of IZ diameter.

Test microorganism	IZ diameter (mm)		Fold increase (%)
	Thymol	Thymol + AgNPs	
Staphylococcus aureus ATCC 43300	28	33	17.9
MR1	31	36	16.1
MR2	31	34	9.7
MR 3	33	37	12.1
MR 4	34	35	2.9
MR 5	30	35	16.7
MR 6	32	33	3.1
MR 7	30	34	13.3
MR 8	29	35	20.7
MR 9	32	35	9.4
MR 10	30	31	3.3
Staphylococcus aureus ATCC 13150	34	38	11.8
MS 1	32	35	9.4
MS 2	32	38	18.8
MS 3	34	36	5.9
MS 4	35	39	11.4
MS 5	33	35	6.1
MS 6	30	34	13.3
MS 7	31	34	9.7
MS 8	33	37	12.1
MS 9	34	38	11.8
MS 10	32	34	6.3

MR: Methicillin resistant *S. aureus;* MS: Methicillin sensitive *S. aureus;* IZ: inhibition zone. Fold increase was calculated using the formula B-A/A*100, where A and B are the zone of inhibition (mm) obtained for thymol alone and in combination with AgNPs, respectively. In case of no inhibition zone, diameter of the disc (6 mm) was taken for the calculation. All experiments were repeated thrice and standard deviations were negligible.

Table 2. MIC of thymol and silver nanoparticles against methicillin-resistant and sensitive *S. aureus* and FICI results using checkerboard technique.

Test microorganism	MIC		Conditions at best synergy point		FICI
	Thymol (µg/ml)	AgNPs (µg /ml)	Thymol (µg/ml)	AgNPs (µg /ml)	
Staphylococcus aureus ATCC 43300	256	80	32	10	0.25 (S)
MR1	128	80	32	20	0.5 (S)
MR2	256	80	32	10	0.25 (S)
MR 3	128	40	32	10	0.5 (S)
MR 4	128	40	64	20	1 (I)
MR 5	256	80	32	10	0.25 (S)
MR 6	128	40	64	20	1 (I)
MR 7	128	80	32	20	0.5 (S)
MR 8	256	160	64	40	0.5 (S)
MR 9	128	80	64	40	1 (I)
MR 10	256	40	128	20	1 (I)
Staphylococcus aureus ATCC 13150	128	40	32	10	0.5 (S)
MS 1	128	20	128	20	2 (I)
MS 2	256	40	64	10	0.5 (S)

Table 2. Contd.

MS 3	128	20	256	40	4 (I)
MS 4	128	40	32	10	0.5 (S)
MS 5	256	80	64	20	0.5 (S)
MS 6	256	40	64	10	0.5 (S)
MS 7	256	40	512	80	4 (I)
MS 8	256	40	64	10	0.5 (S)
MS 9	128	20	32	5	0.5 (S)
MS 10	256	40	64	10	0.5 (S)

MR: Methicillin resistant *S. aureus*; S: synergism; MS: methicillin sensitive *S. aureus;* I: indifference; MIC: minimum inhibitory concentration; FICI: fractional inhibitory concentration index.

DISCUSSION

In 1960, the first strain of MRSA was isolated just one year after the use of methicillin as an alternative to penicillin. Recently, as per the WHO estimates, the incidence of MRSA has reached 70 to 80% of all the *S. aureus* isolates (Jevons, 1961; WHO, 2014). As the battle against super bugs including MRSA rages on, phytochemicals and metallic nanoparticles rise as promising candidates in the combat against MDR pathogens.

In the present study, the phytochemical "thymol" exhibited an inhibitory activity towards both methicillin resistant and sensitive strains of *S. aureus*, whereas silver exhibited negligible activity against either phenotypes when tested by agar diffusion technique. Thyme essential oil and its major phenolic component "thymol" were reported to exhibit a wide spectrum of antimicrobial activity (Dorman and Deans, 2000; Lambert et al., 2001; Friedman et al., 2002; Yap et al., 2014). Investigation of their putative inhibitory efficacy against MSSA and MRSA demonstrated that the susceptibility of the tested strains to thyme oil and thymol was insignificantly different (Nostro et al., 2004). Another study also reported that both methicillin resistant and sensitive strains of *S. aureus* were equally susceptible to thymol and its isomer carvacrol (Nostro et al., 2007). In line with the present results, it has been shown that all *S. aureus* strains were susceptible to thymol whether phenotypically sensitive or resistant to methicillin (Table 1).

While the mechanism of action of essential oil components has not been fully elucidated, most of the components investigated share commonality in their antibacterial mode of action (Nazzaro et al., 2013). Thymol's main mode of action is believed to involve outer and inner membrane disruptions, as well as, binding to intracellular targets disrupting a variety of cellular functions (Walsh et al., 2003; Turina et al., 2006; Xu et al., 2008). Relating the resistance phenotype of microorganisms with the activity of phyto-constituents is conflict ridden. In this regard, limited research has been done for the exploration of the capability of plant extracts in modulating bacterial resistance. Some phytocomponents have been reported to possess resistance modifying activity *in vitro.* Polyphenols in particular have been reported to reverse betalactam resistance in MRSA (Stapleton et al., 2004). Intriguingly, in the present study, there was an insignificant difference in the activity of thymol against both methicillin sensitive and resistant strains of *S. aureus* (Fisher's exact test, $P>0.05$) denoting that thymol activity was not affected by those resistance mechanisms that differentiate these strains (Table 1).

It is documented that a wide range of synthetic components exert antibacterial effects, but the primary impediment for their use is their toxicity. Among these, silver compounds raise as potent bactericidal agents whose medical antimicrobial application has been hindered by their potential toxic effects. Nevertheless, silver at nanoscale shows lower toxicity comparative to conventional silver preparations (Murphy et al., 2015). The evidence supporting AgNPs as effective antimicrobial agents is abundant with reported activity against various bacterial strains including *S. aureus, Staphylococcus epidermidis, Enterococcus faecalis, Enterococcus faecium, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae* and even vancomycin-resistant strains (Ovington, 2004; Panacek et al., 2006; Lok et al., 2007). Disparities in literature, however, occur regarding the antistaphylococcal efficacy of AgNPs. In a study investigating the anti-MRSA effects of different concentrations of biosynthesized silver nanoparticles, it has been reported that the activity was variable with an inhibition zone ranging from 23 to 11 mm and the exerted effect inversely related to the concentrations investigated (Manzoor-ul-Haq et al., 2015). Where silver microbial susceptibility studies have been performed, the authors have assigned their own breakpoints to delineate susceptible and resistant strains. Most of these studies have produced different MIC data for silver, and this demonstrates the extent of variation that currently exists with regard to the pharmacological parameters of silver. For instance, results from the two studies that explored MIC values for *S. aureus* (around 100

strains) ranged from 8 to 80 mg/L (Ug and Ceylan, 2003; Hamilton-Miller et al., 1993). Notwithstanding, the many conflicts in literature regarding the antibacterial activities of AgNPs, it can be conceived that the effect of AgNPs is not solely dependent on nanoparticles characteristics but is also affected by method of assessment used, as well as, the bacterial class tested. In the current study, albeit, the negligible antistaphylococcal effect exerted by silver when tested by agar diffusion technique, AgNPs displayed MIC values ranging from 40 to 160 µg/ml for MRSA and 20 to 80 µg/ml for MSSA strains (Table 2). This may be related to the limited diffusion of AgNPs after disc impregnation and adsorption of certain amounts of AgNPs on the paper disc consequently resulting in lower or non-existent effect. Moreover, it is reported that broth microdilution technique is more reliable and reproducible than disc diffusion technique especially with respect to specific properties of nanoscale materials such as diffusion and aggregation stability in dispersion media (Panacek et al., 2015).

The antibacterial activity of AgNPs is reported to be multifaceted and underlies a decreased probability of resistance development (Franci et al., 2015). Silver nanoparticles were shown to inactivate bacterial enzymes, disrupt bacterial metabolic processes, increase the cytoplasmic membrane permeability, interact with DNA and generate reactive oxygen species that damage biomacromolecules (Lara et al., 2010; Franci et al., 2015). The studies reporting the different mechanisms by which AgNPs interact with microbes paralleled with several reports that investigated the synergistic potential of AgNPs with classical antibiotics (Fayaz et al., 2010; Hwang et al., 2012; Naqvi et al., 2013; Singh et al., 2013). In these studies, quantifying the synergistic effects of antibiotics in combination with AgNPs against an assortment of microorganisms has been investigated, employing the microdilution method. Albeit, the evidenced synergistic capacity of silver nanoparticles in these studies, no trends were observed for the synergistic effects of antibiotics with different modes of action and different chemical structures suggesting that these effects are non-specific.

Recently, several studies have indicated that AgNPs may strengthen the antibacterial effects of antibiotics against both susceptible and resistant bacteria, either additively or synergistically (Fayaz et al., 2010; Lara et al., 2010; Hwang et al., 2012). Consistent with these findings, combining two agents with reported multimodal antibacterial action, in the present study, has shown at least additive or synergistic effect for all tested strains (Table 2). Synchronically, the antibacterial activity of thymol increased when combined with AgNPs as was evident by the fold increase in inhibition zone diameters by disc diffusion method (Table 1). Notably, the resistant strains were inhibited in the combinatorial assays to a greater extent in comparison with when AgNPs and thymol were used in isolation. As per the FICI results, silver concentrations as low as 5 to 20 µg/ml were synergistic and the MIC of thymol was mostly four and eight orders of magnitude lower than those of non-combined thymol (Table 2).

The mechanism by which AgNPs enhanced *S. aureus* sensitivity towards thymol is probably complex taking into account the multiple-level mechanisms reported for both agents. It can be proposed that AgNPs promotes the disturbance of cell wall facilitating thymol's transport into the cell, resulting in disruptions and inactivation of biomacromolecules that has been previously shown to exert. Another interesting finding in the present study is that the synergistic effect of AgNPs in combination with thymol against both resistant and sensitive strains was mostly similar. Influencing resistance mechanisms that differentiate these strains cannot be ruled out but such combination is potentially significant for preventing the development of bacterial resistance. Detailed toxicological studies are needed to establish the safety of the combination regimen of these two agents in the light of decreased effectiveness of classical antibiotics against resistant organisms.

CONFLICT OF INTERESTS

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Mycoflora associated with the goat's hair and sheep wool in Taif, Saudi Arabia

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The objective of this study was to evaluate the occurrence of mycoflora in 30 samples of healthy goat's hair and sheep wool collected from different localities in Taif, Saudi Arabia, and the ability of some fungal isolates for keratinase activities. Sixty four species belonging to 28 genera were collected from the two substrates. The wool of sheep was polluted with fungi than goat hairs, and contained high total counts and number of fungal genera and species. Nine species of true dermatophytes isolated belonged to *Microsporum* (3 species) and *Trichophyton* (6 species). Several keratinophilic species were isolated of which, *Chrysosporium indicum, Chrysosporium keratinophilum* and *Chrysosporium tropicum* were the most prevalent. The commonest saprophytes in order of frequency were members of the genera, *Aspergillus, Penicillium, Alternaria* and *Cochliobolus.* In addition, the other genera found included *Acremonium, Chaetomum, Cladosporium, Cochliobolus, Fusarium, Mucor, Paecilomyces, Phoma, Rhizopus, Scopulariopsis, Stachybotrys, Trichoderma* and others. Five species from 20 tested isolates (*Aspergillus niger, C. keratinophilum, C. tropicum, Microsporum gypseum* and *Trichoderma viride*) had high keratinase activity. The results of this study indicate that both goat hair and sheep wool provide a suitable habitat for dermatophytes and other keratinophilic fungi. Most of these fungi play an important role in the degradation of keratin substrates, so that they can help preserve the environment and reduce pollution.

Key words: Mycoflora, goat's hair, sheep wool, keratinase activities, Saudi Arabia.

INTRODUCTION

Keratinophilic fungi are natural colonizers of keratinous substrates. Some are keratolytic and play an important ecological role in decomposing *α*-keratins, insoluble fibrous proteins (Filipello Marchisio, 2000). Keratinophilic fungi include a variety of filamentous fungi mainly comprising of hyphomycetes and several other taxonomic groups. Hyphomycetes include dermatophytes and a great variety of non dermatophyte filamentous fungi. They occur on cornfield debris in the soil and degrade hard keratin and keratinous material. Therefore, they play an important ecological role in decomposing such residue (Filipello Marchisio, 2000; Sharma and Rajak, 2003).

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Animals are known to carry dermatophytes and other keratinophilic fungi on their hairs. These animals may act as a source of human and animal infections by direct contact or by contaminating working areas and dwelling places (Ripon, 1982). Therefore, the studies on dermatophytes and keratinophilic fungi present on the hair of domestic animals are of considerable significance.

Keratinophilic fungi along with dermatophytes are responsible for various cutaneous mycoses. Dermatophytes require keratin for growth. These fungi can cause different types of tinea in humans and animals. The majority of the fungi responsible for diseases in human beings and animals exist freely in nature as soil saprophytes (Kumari et al., 2005).

The presence of keratinophilic fungi on hairs of various animals has been briefly reviewed by numerous researchers in many parts of the world (Ali-Shtayeh et al., 2000; Dobrowolska et al., 2006; Al-Duboon and Farhan, 2007; Nichita and Marcu, 2010; Sallam and ALKolaibe, 2010; Jain and Sharma, 2012; Emenuga and Oyeka, 2013; Enany et al., 2013).

Keratin is the major component of poultry feathers waste. Distribution of keratinolytic microbes in the nature is widespread. Some fungal strains can produce keratin proteases which have keratolytic activity and can keratinolyse feather. These enzymes have been produced by fungi, including the species of *Aspergillus* spp.*, Cochliobolus* spp.*, Mucor* spp. and *Penicillium* spp. (Friedrich et al., 1999; Soomro et al., 2012; Ramakrishnaiah et al., 2013; Singh, 2014; Singh et al., 2016).

The aim of this investigation was to study the occurrence, distribution and prevalence of mycoflora associated with goat's hair and sheep wool in Taif, Saudi Arabia and the ability of some fungal isolates for keratolytic activity.

MATERIALS AND METHODS

Thirty samples of each of healthy goat's hair and sheep wool were collected from different localities in Taif, Saudi Arabia. The samples were placed in sterile polyethylene bags, transferred immediately to the laboratory, and stored in a refrigerator (3-5°C) until examination. For isolation of mycobiota associated with hair or wool samples, two methods were used:

Hair-baiting technique

For isolation of dermatophytes and other keratinophilic fungi, the hair-baiting technique (Vanbreuseghem, 1952) was employed. Five fragments from each sample were scattered on the surface of moistened sterile soil (20 to 25% moisture content) in sterile plates (3 plates for each sample). The plates were incubated at 25°C for 10-12 weeks and the soil in plates was remoistened with sterile distilled water whenever necessary. The molds which appear on the hair fragments were transferred to the surface of Sabouraud's dextrose agar medium from Sigma (mycological peptone 10.00, dextrose 40.00, agar 15.00) in 1000 ml of distilled water (Moss and McQuown, 1969). The medium was supplemented with 0.5 g cycloheximide (actidione), 40 µg/ml streptomycin and 20 units/ml penicillin as bacteriostatic agents. The plates were incubated at 25°C for 2-4 weeks and the developing fungal colonies were counted, identified (based on morphological and microscopic characters) and calculated per 10 hair fragments for each sample. The relative importance value (RN) was calculated (Ali-Shtayeh and Asad Al Sheikh, 1988; Shearer and Webster, 1985).

The dilution-plate method

The dilution-plate method described by Johnson and Curl (1972) was used for estimation of saprophytic fungi associated with the hair and wool. Czapek Dox Agar medium from Sigma (sucrose 30.0, sodium nitrate 3.0, potassium chloride 0.5, magnesium sulfate heptahydrate 0.5 iron(ii) sulfate heptahydrate 0.01, di-potassium hydrogen phosphate 1.0, Agar 15.0) was used in which rose-bengal (1/15000) and chloramphenicol (25 µg/ml) was added as bacteriostatic agents. Three plates were used for each sample and the plates were incubated at 25°C for 2-3 weeks. The developing fungi were counted, identified and calculated per g hair.

Screening of fungi for keratinase activity

Twenty fungal isolates were screened by using solid medium method (Wawrzkiewiez et al., 1991). Chicken feather was cut into small fragments, washed extensively with water and detergent and dried in a ventilated oven at 40°C for 72 h to prepare feather powder. The feather powder which was the only source of carbon, was added to the sterile agar medium. The diameter of the clear zone was measured after 7 days at room temperature to quantify activity. Keratinase activity of fungus was detected as a clear zone around the colony.

RESULTS AND DISCUSSION

Dermatophytic and keratinophilic fungi (using hair baiting technique)

Sixteen species belonging to 3 genera of dermatophytes and closely related fungi were isolated from goat (13 species and 3 genera) and sheep hairs (14 species and 3 genera). The most contaminated hairs were that of sheep with the higher total counts (226 isolates/300 fragments) and a wide spectrum of species (14 species) than that of goat (174 isolates and 13 species) as shown in Table 1. Similar observations were obtained from goats and sheep in many parts of the world (Nasser and Abdel-Sater, 1997; El-Said et al., 2009; Sallam and ALKolaibe, 2010).

Chrysosporium was the most frequent genus and emerged in 90 and 96.6% of the samples comprising 83.3 and 80.5% of total isolates and have relative importance value RIV of 173.3 and 177.1 of goat and sheep, respectively. This genus was also isolated from goat and sheep hairs in Yemen as reported by Sallam and ALKolaibe (2010), indicating that *Chrysosporium* was recorded in 73 and 68% of the goat hairs and sheep wool samples constituting 57.1 and 47.1% of total fungi, respectively. Also, in Libya, El-Said et al. (2009) observed that *Chrysosporium* was the most frequent genus and emerged in 92 and 96% of the samples comprising 91.2 and 87.8% of the total isolates and have

Table 1. Total isolates (TI, calculated per 300 hair fragments), number of cases of isolation (NCI, out of 30 samples), occurrence remarks (OR) and relative importance values (RIV) of dermatophytic and keratinophilic fungi recovered from hairs of 30 animals of each goats and sheep at 25°C.

	Goats hair			Sheep wool		
	TI	**NCI & OR**	**RIV**	**TI**	**NCI & OR**	**RIV**
Chrysosporium	145	27H	173.3	182	29H	177.1
C. asperatium Carmichael	13	5L	24.1	-	-	
C dermatitidis Carmichael	13	8L	34.1	15	8M	33.3
C. indicum (Randhawa and Sandhau) Garg	15	8M	35.2	17	6L	27.5
C. keratinophilum D. Frey ex J.W. Carmich.	45	12M	65.9	73	19H	95.6
C. pannorum (Link) Hughes	-	-	-	3	2R	8.0
C. tropicum Carmichael	49	10M	61.5	62	14M	74.0
C. xerophilum Pitt	10	6L	25.8	12	4L	18.6
Microsporum	5	3R	12.9	9	7L	27.3
M. canis Bodin	-	-	-	1	1R	3.8
M. ferrugineum M. Ota	-	-	-	2	2R	7.5
M. gypseum (E. Bodin) Guiart & Grigoraki	5	3R	12.9	6	5L	19.3
Trichophyton	24	16H	67.1	35	18H	75.48
T.ajelloi (Vanbreus.) Ajello	2	2R	7.8	-	-	-
T. interdigitale Priestley	4	2R	9.0	8	5L	20.2
T. mentagrophytes (C.P. Robin) Sabour.	7	6L	24.0	5	4L	15.5
T. rubrum (Castell.) Sabour.	2	2R	18.2	7	3R	13.1
T. terrestre Durie & D. Frey	6	5L	20.1	15	7L	36.6
T. tonsurans Malmsten	3	2R	8.4	-	-	
Total isolates		174			226	
Number of genera = 3		3			3	
Number of species= 16		13			14	

Occurrence remarks (OR): H= high occurrence, between 15-30 cases (out of 30); M= moderate occurrence, between 8-14 cases; L= low occurrence, between 4-7 cases; and R= rare occurrence, less than 4 cases.

RIV of 183.2 and 183.8 of goats and sheep, respectively. It was represented by seven species of which *C. keratinophilum , C. tropicum, C dermatitidis* and *C. indicum* were the most prevalent. They emerged in 40.0; 33.0; 26.66 and 26.66% of goat and 63.33; 46.66; 26.66 and 20.0% of samples matching 31.03, 33.8; 9.0 and 10.34%; and 40.1; 34.06; 8.24 and 9.34% of total isolates on the two substrates, respectively. These three species were also, predominant among fungi isolated from cloven hooves and horns of goats and sheep (Abdel-Hafez et al., 1990) and from hairs of goat and sheep (El-Said et al., 2009). The above species were also, isolated from mammals in El-Bahrain by El-Said and Abdel-Sater (1995), Saudi Arabia by Nasser and Abdel-Sater (1997), Bokhary et al. (1999) and Yemen by Sallam and ALKolaibe (2010).

Chrysosporium asperatium and *C. xerophilum* were of low frequency on goats hairs. They were encountered in 16.7 and 20.0% of the samples matching 7.5 and 5.7% of the total isolates. While *C. xerophilum* and *C. pannorum* were of low and rare frequency on sheep hairs. They were encountered in 13.3 and 6.7% of the samples matching 5.3 and 1.3% of the total isolates. The previously identified species was found to be in 4, 1.7, 16 and 12% of goat and sheep hairs in Libya (El-Said et al., 2009). Some species were isolated only from one substrate and not from the other such as *C. asperatium* from goat and *C. pannorum* from sheep hairs (Table 1). Most of these fungi were recovered, with variable degrees and densities from animals hair or natural soil baited, with sterilized human or animals hair from different parts of the world (El-Said, 2002; Periasamy et al., 2004; Dobrowolska et al., 2006; Yahyaraeyat et al., 2009; Beraldo et al., 2011; Emenuga and Oyeka, 2013; Enany et al., 2013; Debnath et al., 2015).

Trichophyton was the next most common genus, and emerged in 53.3 and 60% of the samples comprising 13.8 and 15.5% of the total isolates and have RIV of 67.1 and 75.48 of goat and sheep, respectively (Table 1). It was represented by 6 species of which *T. mentagrophytes, T. terrestre* were of low frequency on goat hairs. They were encountered in 20.0 and 16.6% of the samples matching 4.0 and 3.4% of the total isolates. While *T. interdigitale, T. mentagrophytes* and *T. terrestre* were of low frequency on sheep hairs. They were encountered in 16.6; 13.3 and 23.3% of the samples

matching 2.2; 1.7 and 3.0% of the total isolates. Some species were isolated only from goats such as *T. ajelloi* and *T. tonsurans*, they were isolated with rare frequency (Table 1). This result is in agreement with the finding of Abdel-Hafez et al., (1990) that reported that this genus has rare frequency from cloven hooves and horns of goats and sheep. Also, Bokhary et al. (1999) reported that *T. mentagrophytes* and *T. rubrum* were isolated from goat and sheep in Saudi Arabia. El-Said and Abdel-Sater (1995) noted that *Trichophyton* was represent by 2 species and 1 unidentified species of which *T. rubrum* and *T. terrestre* ware isolated from the two substrates in a rare occurrence. It emerged in 8 and 4% of the samples constituting 2.9 and 3.5% of the total isolates recovered on goats and sheep, respectively. El-Said et al. (2009) reported that *Trichophyton* occurred in 12 and 24% of the animal hair samples examined representing 8.8 and 12.2% of the total isolates and have RIV of 20.7 and 36.1 on goats and sheep, respectively.

Trichophyton was isolated from different animals as reported by several researchers from all over the world (Yahyaraeyat et al., 2009; Nichita and Marcu, 2010; Seker and Dogon, 2011; Jain and Sharma, 2012; Ana Paula et al., 2013; Emenuga and Oyeka, 2013; Enany et al., 2013; Mattei et al., 2014; Debnath et al., 2015; Ilhan et al., 2016; Roshanzamir et al., 2016).

Microsporum was isolated at a low frequency from 10 and 23.3% of the samples, comprising 2.9 and 4% of total isolates and have RIV of 12.9 and 27.3of goat and sheep, respectively (Table 1). It was represented by 3 species (*M. canis; M. ferrugineum* and *M. gypseum*). Some species were isolated only from sheep hairs only *M. canis* and *M. ferrugineum)*. Three species previously mentioned were recovered, with variable degrees and densities from animals in different parts of the world (Sallam and Alkolaibe, 2010; Nichita and Marcu, 2010; Beraldo et al., 2011; Seker and Dogon, 2011; Emenuga and Oyeka, 2013; Mattei et al., 2014; Debnath et al., 2015; Luján-Roca et al., 2016; Roshanzamir et al., 2016).

Saprophytic fungi (using glucose-Czapek's agar)

Forty-eight species belonging to 25 genera were collected from 30 hair samples of each of goats (33 species and 16 genera) and sheep (34 and 20) on glucose-Czapek's agar at 25°C (Table 2). Various saprophytic fungi were encountered and the most prevalent species on the goat sheep were members of *Aspergillus, Penicillium, Alternaria* and *Cochliobolus*. These results are similar to those obtained by El-Said and Abdel-Sater (1995) and El-Said et al. (2009). They reported that members of *Aspergillus, Penicillium, Alternaria* and *Cochliobolus* were the most common in order of frequency, saprophytic fungi from goat and sheep hairs in El-Bahrain and Libya.

Aspergillus was the most common genus, recovered from 56.6 and 66.6% of goat and sheep samples, respectively. From the genus, nine species were identified. *A. niger* was the most prevalent species, although it was isolated at low and moderate frequencies (23.3 and 30%) of goat and sheep samples, respectively. The remaining *Aspergillus* species were less common. *Aspergillus parasiticus* and *Aspergillus terreus* were isolated only from goat hair but were not encountered on sheep hairs. Whilst *Aspergillus ochraceus* and *Aspergillus ustus* were isolated only from sheep hairs but were not encountered on goat hair (Table 2). Mitra et al. (1998) noticed that *Aspergillus* species were the most common among fungi other than dermatophytes isolated from the goats and sheep. Also, Gherbawy et al. (2006) noticed that *Aspergillus* (7 species +1 variety) was the first most dominant fungi on human hairs in Upper Egypt. Additionally, most of *Aspergillus* species were previously isolated in Gaza from the sheep (Abdel-Hafez, 1987), as well as hairs of animals in many parts of the world (Sanches and Coutinho, 2007; Blyskal, 2009; Sallam and AL-Kolaibe, 2010; Nichita and Marcu, 2010; Luján-Roca et al., 2016).

Penicillium (5 species) was the second most predominant genus and occurred in 43.3% of goat hairs contributing 24.4% of the total moulds. *P. chrysogenum* was the most prevalent species, although it was isolated at moderate frequencies (26.6%) of goat samples. The remaining *Penicillium* species were less common and were isolated in rare frequencies (Table 2). While *P. chrysogenum* and *P. corylophilum* were isolated in low and rare frequencies, occurring in 16.66 and 3.33% of sheep hairs, and contributing 4.1 and 1.36% of the total moulds, respectively (Table 2). *Penicillium brevicompactum, P. purpurogenum* and *P. variabile* were isolated only from goat hair but were not encountered on sheep hairs (Table 2). Additionally, members of *Penicillium* were among the most common fungi on the hair of different animals from Libya (El-Said et al., 2009), Saudi Arabia (Nasser and Abdel-Sater, 1997), Romania (Nichita and Marcu, 2010) and Yemen (Sallam and AL-Kolaibe, 2010).

Alternaria (*A. alternate*, *A. raphani* and *A. tenuissima*) was isolated in low and moderate occurrence from one or two substrates. They were found in 13.3-26.6% of the samples tested comprising 7.3-13.7% of total isolates on goats and sheep, respectively. While *Cochliobolus* (*Cochliobolus australiensis, Cochliobolus lunatus* and *Cochliobolus spicifer*) was isolated in low occurrence from sheep. Other species of the preceding genera were less frequent (Table 2). The remaining genera were isolated in rare or low frequencies of occurrence and were encountered collectively in 1.2-7.3% and 1.4-6.8% of the total molds on goat and sheep hairs, respectively (Table 2).

Some fungi were isolated only from goat hairs, which include *Acremonium kiliense, Aspergillus parasiticus, A. terreus, Chaetomium spirale, Cladosporium cladosporioides, Curvularia lunata, Geotrichum candidum, Paecilomyces*

Table 2. Total counts (TC), percentage frequency (%F, calculated per 30 samples, numbers of cases of isolation (NCI) and occurrence remarks (OR) of various fungal genera recovered from hairs of 30 animals of each of goats and sheep on agar at 25°C.

Genera and species	Goats hair			Sheep wool		
	TC	F (%)	NCI & OR	TC	F (%)	NCI & OR
Acremonium	2	6.6	2R	1	3.3	1R
A. strictum W. Gams	1	3.3	1R	1	3.3	1R
A. kiliense Grütz	1	3.3	1R	-	-	-
Alternaria	6	13.3	4L	10	26.6	8M
A. alternata (Fr.) Keissl.	4	10	3R	6	20	6L
A. raphani J.W. Groves & Skolko	-	-	-	1	3.3	1R
A. tenuissima (Kunze) Wiltshire	2	6.6	2R	3	6.6	2R
Aureobasidium pullulans (de Bary & Löwenthal) G. Arnaud	-	-	-	1	3.3	1R
Aspergillus	24	56.6	17H	26	66.6	20H
A. flavus Link	3	6.6	2R	3	10	3R
A. fumigatus Fresenius	1	3.3	1R	2	6.6	2R
A. ochraceus G. Wilh.	-	-	-	2	6.6	2R
A. parasiticus Speare	1	3.3	1R	-	-	-
A. niger sensu auct. pro parte, pre	10	23.3	7L	12	30	9M
A. sydowii (Bainier & Sartory) Thom & Church	4	13.	4R	2	6.6	2R
A. terreus Thom	2	6.6	2R	-	-	-
A. ustus (Bainier) Thom & Church	-	-	-	4	10	3R
*A. variecolo*r Thom & Raper	3	6.6	2R	1	3.3	1R
Botryotrichum piluliferum Sacc. & Marchal	-		-	1	3.3	1R
Chaetomium	2	6.6	2R	1	3.3	1R
C. globosum Kunze	1	3.3	1R	1	3.3	1R
C. spirale Zopf	1	3.3	1R	-	-	-
Cladosporium cladosporioides (Fresen.) G.A. de Vries	2	3.3	1R	-	-	-
Cochliobolus	4	10	3R	9	16.6	5L
C. australiensis (Tsuda & Ueyama) Alcorn	-	-	-	3	10	3R
C. lunatus R.R. Nelson & F.A. Haasis	2	3.3	1R	1	3.3	1R
C. spicifer R.R. Nelson	2	3.3	2R	5	10	3R
Curvularia lunata (Wakker) Boedijn	1	3.3	1R	-	-	-
Emericella nidulans (Eidam.) Vuillemin	-	-	-	1	3.3	1R
Eurotium amstelodami Mangin	1	3.3	1R	3	6.6	2R
Fusarium	4	10	3R	4	10	3R
F. oxysporum Schltdl.	2	6.6	2R	1	3.3	1R
F. solani (Mart.) Sacc.	2	3.3	1R	3	10	3R
Geotrichum candidum Link	1	3.3	1R	-	-	-
Myrothecium verrucaria (Alb. & Schwein.) Ditmar	-	-	-	2	6.6	2R
Mucor hiemalis Wehmer	-	-	-	1	3.3	1R
Paecilomyces lilacinus (Thom) Samson	2	3.3	1R	-	-	-
Penicillium	20	43.3	13H	4	13.3	4L
P. brevicompactum Dierckx	2	6.6	2R	-	-	-
P. chrysogenum Thom	11	26.6	8M	3	16.6	5L
P. corylophilum Dierckx	5	10	3R	1	3.3	`1R
P. purpurogenum Stoll	1	3.3	1R	-	-	-
P. variabile Sopp	1	3.3	1R	-	-	-
Phoma	3	10	3R	1	3.3	1R
P. herbarum Westend.	2	6.6	2R	1	3.3	1R
P. glomerata (Corda) Wollenw. & Hochapfel	1	3.33	1R	-	-	-

Table 2. Contd.

Genera and species	Goats hair			Sheep wool		
	TC	F (%)	NCI & OR	TC	F (%)	NCI & OR
Pleospora herbarum P. Karst.	-	-	-	1	3.3	1R
Rhizopus nigricans Ehrenb.	1	3.3	1R	-	-	-
Scopulariopsis	6	6.6	2R	3	10	3R
S. brevicaulis (Sacc.) Bainier	4	10	3R	2	6.6	2R
S. brumptii Salv.-Duval	2	3.3	1R	-	-	-
S. candida Vuill.	-	-	-	1	3.3	1R
Stachybotrys chartarum (Ehrenb.) S. Hughes	-	-	-	1	3.3	1R
Stemphylium botryosum Wallr.	-	-	-	1	3.3	1R
Sterile mycelia	2	6.6	2R	3	6.6	2R
Trichoderma viride Pers.	-	-	-	1	3.3	1R
Ulocladium	1	3.3	1R	5	10	3L
U. alternariae (Cooke) E.G. Simmons	-	-	-	2	6.6	2R
U. chartarum (Preuss) E.G. Simmons	1	3.3	1R	3	6.6	2R
Total count		82			73	
Number of genera = 25		16			20	
Number of species= 48		33			34	

Activity remarks H= high, M= moderate, L= low keratinase activity. Occurrence remarks (OR): H= high occurrence, between 15-30 cases (out of 30); M= moderate occurrence, between 8-14 cases; L= low occurrence, between 4-7 cases; and R= rare occurrence, less than 4 cases.

lilacinus, Penicillium brevicompactum, P. purpurogenum, P. variabile, Phoma glomerata, Rhizopus nigricans and *Scopulariopsis brumptii*. While *Alternaria raphani, Aureobasidium pullulans, Aspergillus ochraceus, Aspergillus ustus, Botryotrichum piluliferum, Cochliobolus australiensis, Emericella nidulans, Myrothecium verrucaria, Mucor hiemalis, Pleospora herbarum, Scopulariopsis candida, Stachybotrys chartarum, Stemphylium botryosum, Trichoderma viride* and *Ulocladium alternariae* were isolated only from sheep wool (Tables 1 and 2). These results agree to some extent with some findings (Ogawa et al., 2008; Nichita and Marcu, 2010; Sallam and Alkolaibe, 2010; Emenuga and Oyeka, 2013; Luján-Roca et al., 2016).

Keratinase activity of the isolated fungi

Data in Table 3 and Figure 1 show that the high keratinase activity as a clear zone around the colony (54, 65, 50, 58 and 60 mm) were observed for *A. niger, Chrysosporium keratinophilum, C. tropicum, Microsporum gypseum* and *Trichoderma viride,* respectively. While *Acremonium strictum, Fusarium oxysporum, Myrothecium verrucaria, Mucor hiemalis, Scopulariopsis brevicaulis* and *Trichophyton terrestre* had moderate keratinase activities (45, 43, 32, 45, 45 and 34mm), respectively. On the other hand, the *C. dermatitidis, C. cladosporioides, P. chrysogenum,*

Table 3. Keratinase activity as clear zone (MM) of dermatophytes and other keratinophilic fungi.

Fungal isolates	Clear zone (MM)
Acremonium strictum	45M
Alternaria alternata	0
Aspergillus niger	54H
Chrysosporium dermatitidis	25L
C. keratinophilum	65H
C. tropicum	50H
Cladosporium cladosporioides	18L
Curvularia lunata	0
Fusarium oxysporum	43M
Microsporum gypseum	58H
Myrothecium verrucaria	32M
Mucor hiemalis	45M
Penicillium chrysogenum	20L
Scopulariopsis brevicaulis	45M
Trichoderma viride	60H
Trichophyton interdigitale	25L
T. mentagrophytes	15L
T. rubrum	23L
T. terrestre	34M
Ulocladium chartarum	21L

Trichophyton interdigitale, T. mentagrophytes, T. rubrum and *Ulocladium chartarum* were low keratinase activities

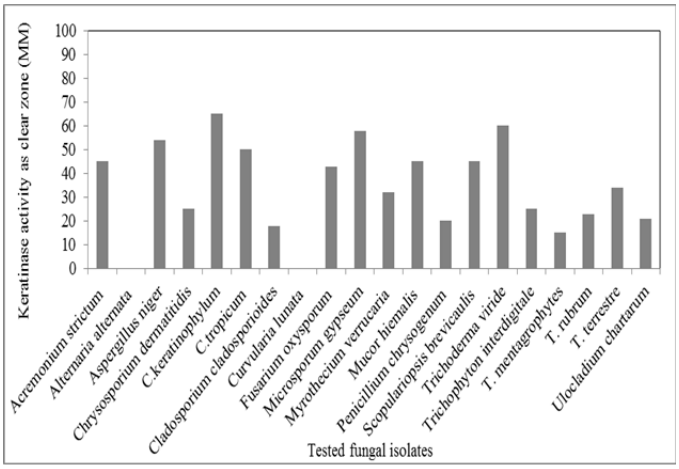

Figure 1. Keratinase activity as clear zone (MM) of dermatophytes and other keratinophilic fungi.

(25, 18, 20, 25, 15, 23 and 21 mm), respectively. While *Alternaria alternate* and *Curvularia lunata* had negative keratinase activity

The results agree to some extend with previous findings (Anbu et al., 2008; Awasthi and Kushwaha, 2011; Ramakrishnaiah et al., 2013; Kumar and Kushwaha, 2014; Singh, 2014; Singh et al., 2016; Bohacz, 2017).

Thus, the study concluded that members of *Chrysosporium* and dermatophytes (*Trichophyton* and *Microsporum*) were consistently the most frequent fungi on the goats' hairs and sheep wool. Also, various saprophytic and cycloheximide resistant fungi were common on goat and sheep hairs tested and these were members of *Acremonium, Alternaria, Aspergillus, Chaetomium, Cochliobolus, Chdaetomium, Fusarium, Penicillium* and *Scopulariopsis.* The wool of sheep was contaminated more than the goat's hairs. This may be due to the increase of organic in sheep hair than goat hair. Most dermatophytes and other keratinophilic fungi play an important role in the degradation of keratin substrates, so that they can help preserve the environment and reduce pollution.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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Evaluation of safety and immunogenicity of inactivated whole culture contagious caprine pleuropneumonia trial vaccine in National Veterinary Institute, Ethiopia

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Contagious caprine pleuropneumonia (CCPP) is a fatal disease of goats occurring in many countries of Africa and Asia where the total goat population is more than 500 million. Vaccination is the most cost effective technique in the control of CCPP than any other control measures. In National Veterinary Institute (NVI) inactivated mycoplasma protein based vaccine obtained by centrifugation has been in use since many years. This study focuses on evaluating the safety and immunogenicity of inactivated whole culture CCPP vaccine currently developed in the NVI. Twenty six *Mycoplasma capricolum subspecies capripneumoniae* (Mccp) antibody free goats were used to evaluate the safety and immunogenicity of inactivated whole culture CCPP trial vaccine. The trial vaccine was prepared from culture of Mccp vaccinal seed grown in Mycoplasma specific hayflick media using spinner bottle. The protein content for one milliliter of whole culture was checked and found to be more than the minimum recommended dose (0.15 mg per dose). The culture was inactivated by 37% formalin at proportion of 0.5% of whole culture and adjuvanted by saponin at final concentration of 0.3%. The experimental animals were distributed into four groups: The group A consist of five goats for safety and all the other groups consists of seven animals each with group B for trial vaccine, group C for positive control and group D for negative control for immunogenicity trials. The goats were observed for two months for safety and immunogenicity evaluation during which serum samples were collected for immunogenicity and tested by using competitive Enzyme Linked immunosorbent Assay (cELISA) test. The results indicated that out of 7 goats vaccinated with trial vaccine, the mean sero-positivity was 60.71% while 7 goats vaccinated with the positive control showed mean sero-positivity of 58.86%. The analysis showed no significant difference between mean sero positivity of trial vaccine and positive control (P>0.05) as indicated by sero-conversion. The mean percent inhibition (PI) of trial inactivated whole culture CCPP vaccine vaccinated goats was 61.52% while the mean PI for positive control vaccine vaccinated group was 51.86%. In contrast the non-vaccinated controls showed mean PI of 40.65% which is significantly less than percent inhibition of the vaccinated groups (p=0.000). The body temperature and clinical observation of safety tested animals and other immunogenicity tested goats showed absence of any abnormality after vaccination both in vaccinated and controls. This study which was novel in its nature concluded that the trial inactivated whole culture ccpp vaccine is equally immunogenic as that vaccine already in use, the non-whole culture concentrated CCPP vaccine, and could be used for mass vaccination after conducting field immunogenicity trial.

Key words: Inactivated whole culture ccpp vaccine/goats/spinner bottle/safety/immunogenicity/NVI

INTRODUCTION

Contagious caprine pleuropneumonia (CCPP) is a severe disease of goats occurring in many countries of Africa and Asia where the total goat population is more than 500 million (Acharya, 1992). A classical, acute CCPP is caused by Mycoplasma capricolum sub species .Capripneumoniae originally known as the F38 biotype (Mccp) (Leach et al., 1993; Mac Martin et al., 1980). Typical cases of CCPP are characterized by extreme fever (41 to 43°C), high morbidity and mortality rates in susceptible herds affecting all ages and both sexes. The clinical symptoms of the disease include abortion of pregnant goats, accelerated and painful respiration sometimes accompanied by a grunt, frequent, violent and productive coughing and in the terminal stages the goats are unable to move making them stand with their front legs wide apart, stiff and extended neck. The post-mortem examination of affected animals reveals fibrinious pleuropneumonia with massive lung hepatisation and pleurisy, accompanied by accumulation of straw-colored pleural fluid (OIE, 2014)

CCPP was first reported in Europe in 2004(Ozdemir et al., 2005) while the presence of CCPP in Ethiopia had been suspected since 1983 and confirmed later in1990 by isolation and identification of Mccp from the outbreak of CCPP in Ogaden, eastern Ethiopia (Thiaucourt et al., 1992). Since then the disease has been known to be endemic in different regions of the country (Sharew et al., 2005). Outbreaks of CCPP have been reported from almost all regions of the country especially from low land areas which are known as goat rearing regions (APHRD, 2010). Despite the presence and economic importance of CCPP in Ethiopia, the exact prevalence and distribution in the country has not been studied yet. However, few prevalence studies report showed CCPP prevalence as 31.6% by Lakewu et al. (2014) in Borana pasteural area, 4.92% by Yousuf et al. (2012), Dire Dawa, 32.36% by Sherif et al. (2012) in JigJiga. This indicates that the CCPP is one of the serious problem affecting goats population in the studied areas.

The disease can be controlled by the use of inactivated CCPP vaccine that has the ability to protect goats from infection for fourteen months (OIE, 2014; Litamoi et al., 1989). Despite the efficacy of this vaccine in Ethiopia, there are some constraints like the fastidious nature of the Mccp seed, less advanced technology used, the slow process of centrifugation or concentration employed to produce the existing CCPP vaccine makes Ethiopia not to satisfy its customers' demand with regard to CCPP vaccine production. Most studies tried so far did not bring promising output to address this problem. However, some pilot study made at NVI indicated the possibility of getting adequate antigen per millimeter of whole culture of Mccp seed which urges us to launch this study to see the safety and immunogenicity of whole culture CCPP vaccine. Therefore, this study was designed with aim to evaluate the safety and immunogenicity of whole culture CCPP inactivated vaccine in comparison to the existing CCPP inactivated vaccine produced by concentration of cultural antigen.

MATERIALS AND METHODS

Study area

This experimental study was conducted from September 2014 to April 2015 at National Veterinary Institute in Bishoftu town which is situated 47 km south east of the capital city, Addis Ababa. It was found at 9°N latitude and 4° E longitudes at an altitude of 1850 m above sea level in central highlands of Ethiopia (NMSA, 1999). National veterinary Institute is the sole veterinary vaccine producing laboratory in Ethiopia and currently it produces over 150 million doses of veterinary vaccines each year.

Antibody screening and husbandry of experimental animals

Twenty six male goats older than six months of approximately equal age with history of no previous exposure to CCPP and negative for Mccp specific antibodies were used for this experiment. Clinically healthy goats were purchased from local live- stock market and were kept in clean clear sheds. All the animals were screened for Mccp antibody by using c-ELISA. The animals declared negative for CCPP were treated by Albendazole and oxytetracycline and left for two weeks for adaptation. They were offered appropriate feeds like wheat bran, maize and Alfalfa, and clean water ad libitum.

Experimental design

Culture preparation

New stock of Mccp seed was obtained from vaccine seed bank (NVI) and passaged three times at weekly interval in hayflick media. The culture showing adequate change in PH (6.65-6.95) and free from contamination was allowed to pass to the next passage. Inoculum from the last passage was inoculated to production media in proportion of: 20% seed culture, 20% horse sera and 60% hayflick media. The mixture was incubated with continuous slow agitation in spinner bottle (90-100 rpm) at 37°C for 7 to 10 days until the desired turbidity and pH (6.4-6.8) was achieved. Sample was taken aseptically, smeared on slide, stained and observed for presence of any contaminant before proceeding to the next step (OIE, 2014).

Protein estimation for the whole culture

To estimate the protein content of the whole culture serious steps

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were carried out: the Mccp culture with adequate level of growth having optimum pH and purity was inactivated by 0.5% formalin and homogenized by shaking. Then, 100 ml Mccp culture was taken as sample and centrifuged at 20,000 rpm during which the supernatant was discarded and the pellet was washed three times to remove remnant of media. The amount of Mccp antigen was determined for this 100 ml sample and checked whether it had sufficient antigen (great or equal to 0.15 mg per ml of whole culture) by using bicinchoninic acid (BCA) assay. In this BCA assay 50 µl of known Bovine serum albumin (BSA) standards and 50 µl test samples (Mccp pellet suspension) and BCA and Copper sulphate were used and mixed as follows. Triplicate 50 µl of BSA and pellet suspension were each half fold diluted by using phosphate buffered saline (PBS), and 150 µl of bicinchoninic acid Cupper (II) sulphate (CuSO4) mix (in proportion of 50:1) was added to each well of U-shaped micro-plate. The plate was then incubated for 1 h at 37°C, and then OD of the reaction was read in ELISA reader at 562 nm wavelength. The regression line formula was drawn from average OD of the standard, and average protein concentration was calculated for the pellet suspension of Mccp inactivated whole culture from the regression line formula.

Adjuvant preparation

Saponin (Quillaja saponin, Guiness product) stock solution was prepared in 10% concentration with PBS and sterilized by steam autoclaving at 121°C for 15 min. The sterility of this stock saponin solution was checked by culturing it on .SBCDM agar and tryptose soya broth, thioglycollate broth and sabouraud agar media.

CCPP Inactivated whole culture trial vaccine formulation

The Mccp culture that had protein antigen content greater or equal to 0.15 mg/ml of whole culture was adjuvanted by adding saponin to make final concentration of saponin to 0.3% and then dispensed in 100 ml volume in polypropylene vials. The prepared vaccine was subjected to safety and other quality control tests (OIE, 2014).

CCPP inactivated whole culture trial vaccine quality control

Purity: The formulated vaccine was checked for presence of contaminants, by culturing a sample from the formulation on sterility test media including SBCDM agar and tryptose soya broth, thioglycollate broth and sabouraud agar media. The samples were also checked by Gram staining for bacterial contaminants.

Inactivation test: For inactivation test ten tubes marked from 1 to 10 each containing 10 ml of hayflick media were taken. A volume of 1 ml of inactivated whole culture was inoculated to four tubes while four tubes were inoculated with live *Mccp* inoculums as a positive control and the other two tubes were kept as negative control containing only media. All the tubes were incubated at 37°C for 10 days and observed for the presence or absence of mycoplasma growth.

Safety: Safety of the trial vaccine was checked by injecting three goats with 2 ml by subcutaneouse route and 2 goats were kept as control. The vaccinated goats were examined daily for presence of disease symptoms and injection site reaction and rectal temperature was recorded twice daily for 14 days at 9:am and 3:pm.

Experimental animals grouping and vaccination

After two weeks observation period, the experimental animals (n=26) which were Mccp antibody free were randomly allocated into four experimental groups as follows: Group A consists of five (n=5) goats which were used to evaluate the safety of the whole culture trial vaccine, group B consists of seven (n=7) goats which were vaccinated with trial whole culture inactivated CCPP vaccine and group C consists of seven (n=7) goats which were vaccinated with CCPP vaccine which was used as positive controls. On the other hand the remaining seven goats (n=7) where left as non-vaccinated controls in group D being injected with 1 ml sterile hayflick media (as placebo).

Follow up of vaccinated goats and blood sampling

Once the animals were vaccinated for immunogenicity test, the rectal temperatures of study goats were recorded for 7 days twice daily at 9:am and 3:pm. 5 ml blood was collected in vacutainer tube without EDTA from each experimental goats once per week at days 7, 14, 21, 28, 35, 42,49 and 56 of post vaccination. The blood samples were allowed to stand for 30 to 45 min and centrifuged at 3000 rpm for 3 min to extract clear serum (Tuck et al., 2009). The collected serum was stored at -20°C until processing for sero conversion by using c-ELISA test method (IDEXX, product) at Research and Development laboratory of NVI, Ethiopia.

Data analysis

The data regarding body temperature follow up and immunogenicity parameters were entered in Microsoft office Excel computer program and then summarized first by using descriptive statistics. Further statistical analysis was performed using statistical package for social science (SPSS)-version 20. The post-vaccination sero-conversion per group was calculated as the proportion of cELISA positive animals to the total test animals per group as percentage. Comparison of the rectal temperature data of safety tested goats and the mean percentage sero-conversion in different groups was done by using independent sample t-test. The mean percent inhibition of sero-conversion of immunogenicity trial groups, the temperature data of immunogenicity tested groups and the mean weekly percent inhibition of sero-conversion were compared by using analysis of variance (ANOVA). In all the analyses, confidence interval was at 95% and desired level of precision was 0.05.

RESULTS

Safety test

During these all observation periods, the animals showed no abnormal situation except minor swelling at the injection site which subsided within a week. The body temperature recorded for safety tested goats was analyzed and it showed that the body temperature remained within recommended range for healthy goats (37.5 to 40.5°C) and there was absence of significant difference between the vaccinated and non-vaccinated goats (P>0.05) (Tables 1 and 2). Besides these five animals, the three groups tested for immunogenicity were also checked for their body temperature difference for the first 7 days after vaccination. The temperature of both control and inoculated animals was almost same and no significant difference (P>0.05) was found as shown in

Table 1. Mean morning body temperature of experimental animals inoculated with newly prepared vaccine and control groups kept for14 days observation for safety test.

Group	n	mean	Min	max	STD	SE	Tcal	pvalue
Test goat morning temperature	14	38.31	37.23	39.37	0.517	0.138	1.754	0.091
Control goats morning temperature	14	37.94	36.65	38.70	0.575	0.154		

n, number of days temperature is observed; degree of freedom (df)=26; Min and max, minimum and maximum observed value respectively; STD, standard deviation; SE, standard error. The mean difference is significant at p-value ≤ 0.05; Normal body temperature of goats lies between 37.5 and 40.5°C.

Table 2. Mean afternoon body temperature of experimental animals inoculated with newly prepared vaccine and control groups kept for14 days observation for safety test.

Group	n	Mean	Min	Max	STD	SE	t-cal	p-value
Test goat afternoon temperature	14	39.67	39.4	40.03	0.196	0.052	0.061	0.952
Control goats afternoon temperature	14	39.68	39.05	41.05	0.548	0.146		

n, number of days temperature is observed; degree of freedom (df)=26; Min and max, minimum and maximum observed value respectively; STD, standard deviation; SE, standard error; The mean difference is significant at p-value ≤ 0.05; Normal body temperature of goats lies between 37.5 and 40.5°C.

Table 3. Mean morning body temperature of immunogenicity tested and control group animals.

Group	n	Mean	Min	Max	STD	SE	Fcal	p-value
Whole culture vaccinated goat	7	38.5	38.36	38.96	0.21	0.079		
Positive control vaccinated goats	7	38.49	38.33	38.61	0.09	0.035	0.233	0.794
Non-vaccinated Control goats	7	38.42	38.03	39.09	0.33	0.123		

n is number of days for which body temperature was taken; df1 = 2, df2 = 18; Min and max, minimum and maximum observed value respectively; STD, standard deviation; SE-standard error.; mean difference is significant at p ≤ 0.05.

Table 4. Mean afternoon body temperature of immunogenicity tested and control group animals.

Group	n	Mean	Min	Max	STD	SE	F-cal	p-value
Whole culture vaccinated goat	7	39.44	38.86	40.10	0.446	0.169		
Positive control vaccinated goats	7	39.58	39.39	39.94	0.189	0.0718	1.549	0.239
Non-vaccinated Control goats	7	39.74	39.29	40	0.264	0.099		

n is number of days for which body temperature was taken; df1=2, df2=18; Min and max, minimum and maximum observed value respectively; STD, standard deviation; SE-standard error; mean difference is significant at p ≤ 0.05.

Tables 3 and 4.

Immunogenicity test

For the period of eight weeks sero-conversion, ability of goats categorized under the three treatment groups were analyzed. Accordingly, the comparison of mean percent positive of sero-conversion for eight weeks duration of trial vaccine and positive control and also comparison of mean percent inhibition for eight weeks in the three groups were given in Tables 5 and 6.

As the above PI comparison showed presence of significant difference (P< 0.05) among the three treatment groups, to know between which groups significant difference exist, multiple comparison was made (in post hoc analysis of ANOVA) and the result is given as follows (Table 7).It showed that mean PI of whole culture is the highest (61.52±2.92) and the mean PI of positive control (51.86±4.95) is greater than that of negative control (40.65±2.84).

The above pair wise comparison by ANOVA indicated that the presence of significant difference among the mean PI value in the test groups. This means, there was

Table 5. Mean percent positives for three different treatment groups.

Vaccination group	N	Mean	Std. Dev.	SE	95% CI	Min	Max	t-cal	p-value
Positive control	8	58.86	19.26	6.8	42.76-74.96	42.86	85.71	0.258	0.803
Whole culture	8	60.71	6.61	2.3	55.18-66.24	57.14	71.43		
Negative controls	8	0	0	0	-	-	-	-	

N=8 for all groups and is the number of weeks when goats were observed; CI-confidence interval; df = 8.629; Min and max, minimum and maximum observed value respectively; STD-standard deviation; SE, standard error; The comparison of the mean difference of percentage positives between whole culture vaccinated (60.71%) and positive control vaccinated (58.86%) showed absence of significant difference between the two groups (P>0.05).

Table 6. Mean percentage inhibition of sera of the three treatment groups taken for eight weeks.

Group	Mean	Std. Dev.	SE	95% CI	Min	Max	F-statistic	p-value
Positive control	51.86	4.95	1.75	47.72-55.99	45.94	57.95		
Whole culture	61.52	2.92	1.03	59.07-63.96	55.44	64.20	100.056	0.000
Negative controls	40.65	2.84	1.00	38.28-43.02	36.66	44.63		

N=8 for all groups and is the number of weeks goats were observed; CI-confidence interval; df1 = 2, df2 = 13.459: Min and max, minimum and maximum observed value respectively; STD, standard deviation; SE, standard error.

Table 7. Comparisons of the three treatment groups with each other by their mean percent inhibition (PI) showing point of significant difference between mean PIs.

Group (I)	Group (J)	Mean difference (I-J)	SE	p-value
Positive control	Whole culture	-9.661	2.03	0.001
	Negative control	11.206	2.02	0.000
Whole culture	Positive control	9.661	2.03	0.001
	Negative control	20.867	1.44	0.000
Negative control	Positive control	-11.206	2.02	0.000
	Whole culture	-20.867	1.44	0.000

Mean difference is significant at level of ≤ 0.05; SE is standard error.

strong significant difference between whole culture vaccinated and positive control vaccinated where the PI of whole culture vaccinated was greater than the PI of positive control vaccinated and the PI of non-vaccinated controls was significantly less than both vaccinated groups. After this observation that means PI was significantly different among the three treatment groups (Tables 6 and 7). Table 8 shows weekly summary statistics for PIs for three treatment groups for 8 weeks observation periods.

When the above mean weekly observation is depicted graphically the mean percentage inhibition of the whole culture was above both the mean percentage inhibition of positive and negative controls in all weeks. In some cases, the mean percentage inhibition of positive control failed below cut off value of c-ELISA (<50) but the mean Percentage inhibition of the negative control was as expected (below 50 throughout the weeks when the data was collected) (Figure 1).

DISCUSSION

In Ethiopia goat rearing has got important means of income especially in pastoral areas where goats are reared in large numbers (Hirpa and Abebe, 2008). Despite their importance goats have been known to be affected by tremendous infectious diseases which cost their life and contribution. CCPP is one of the most important infectious disease affecting goats contributing for high mortality and economic loss.

The main objective of this experimental study was to evaluate the safety and immunogenicity of inactivated whole culture CCPP trial vaccine in comparison to the

Table 8. Mean percentage inhibition and summary statistic of seroconversion for different treatment groups on weekly basis for eight weeks.

Group		N	Mean	Std. deviation	Std. error	95% Confidence interval for mean		Minimum	Maximum
						Lower Bound	Upper Bound		
	Positive controls	7	57.40	8.64	3.27	49.41	65.40	41.25	70.26
PIW1	Whole culture	7	63.02	23.73	8.97	41.07	84.98	26.84	93.95
	Non vaccinated	7	43.37	5.90	2.23	37.92	48.84	34.48	51.93
	Positive controls	7	57.71	12.14	4.59	46.48	68.93	39.38	77.70
PIW2	Whole culture	7	63.95	23.13	8.74	42.56	85.35	36.37	89.76
	Non vaccinated	7	44.63	11.64	4.40	33.87	55.40	27.83	66.90
	Positive controls	7	48.64	9.77	3.69	39.62	57.66	35.62	67.53
PIW3	Whole culture	7	61.62	21.52	8.13	41.72	81.52	34.11	89.76
	Non vaccinated	7	41.88	5.14	1.94	37.13	46.63	33.23	47.80
	Positive controls	7	57.95	10.30	3.89	48.4224	67.48	48.14	74.59
PIW4	Whole culture	7	64.20	21.90	8.28	43.94	84.45	36.82	94.27
	Non vaccinated	7	40.15	7.85	2.97	32.89	47.41	27.32	49.30
	Positive controls	7	45.96	8.54	3.23	38.066	53.86	32.76	55.84
PIW5	Whole culture	7	59.40	23.98	9.06	37.22	81.57	32.76	93.63
	Non vaccinated	7	40.11	7.27	2.75	33.38	46.87	29.71	53.45
	Positive controls	7	49.79	10.18	3.84	40.37	59.21	32.61	62.55
PIW6	Whole culture	7	61.27	21.66	8.19	41.24	81.30	32.23	91.97
	Non vaccinated	7	36.66	7.32	2.77	29.89	43.43	24.40	44.03
PIW7	Positive controls	7	48.69	13.36	5.05	36.33	61.0492	23.87	63.00
	Whole culture	7	55.44	20.01	7.56	36.93	73.95	29.18	84.75
	Non vaccinated	7	36.86	5.03	1.90	32.21	41.52	29.84	43.10
	Positive controls	7	48.74	11.16	4.22	38.41	59.07	27.84	61.89
PIW8	Whole culture	7	63.25	17.47	6.60	47.09	79.41	42.89	90.75
	Non vaccinated	7	41.54	6.71	2.54	35.34	47.74	28.00	48.32

PIW1, PIW2, PIW3, PIW4, PIW5, PIW6, PIW7 and PIW8 indicates mean percentage inhibition of week 1 to 8, respectively.

inactivated CCPP vaccine which has been produced through other technique in NVI. Accordingly, trial inactivated whole culture CCPP vaccine was prepared based on standard operational procedure of CCPP vaccine production in NVI (Tesgera and Tefera, 2012; OIE, 2014) and the trial vaccine was subjected to all the quality control checks which were made according to OIE (2014). The safety test of this trial vaccine was made on goats prior to the commencement of the immunogenicity test. Analysis of rectal temperature for safety tests for 14 days twice daily showed no significant difference between the vaccinated and the control groups ($p > 0.05$) and these were within the normal goat body temperature (Radosits et al., 2007). This was also of the case for body temperature of immunogenicity tested animals where there was no significant difference between body temperature of whole culture vaccinated, positive control vaccinated and non-vaccinated controls ($P > 0.05$). It has been recommended that for the vaccine to pass safety tests, animals must not show significant abnormalities except minor swelling at the injection site which should subside within a week of observation (OIE, 2014). Hence, the current experiment proved the trial vaccine to be safe. The mean weekly sero-conversion (Mccp antibody positivity) of goats in three groups indicated that the whole culture vaccinated group had 60.71% (n=7) goats sero-positive, positive control vaccinated group had 58.86% (n=7) goats positive while the non-vaccinated control group showed no sero-positivity throughout two months of observation The sero-positivity between whole culture vaccinated group and positive control vaccinated group showed no significant difference ($P > 0.05$). This result indicated that the new trial vaccine has comparable merits to the existing NVI inactivated CCPP vaccine prepared by concentration method. Lakew et al. (2014) reported rise in the seropositivity after vaccination with

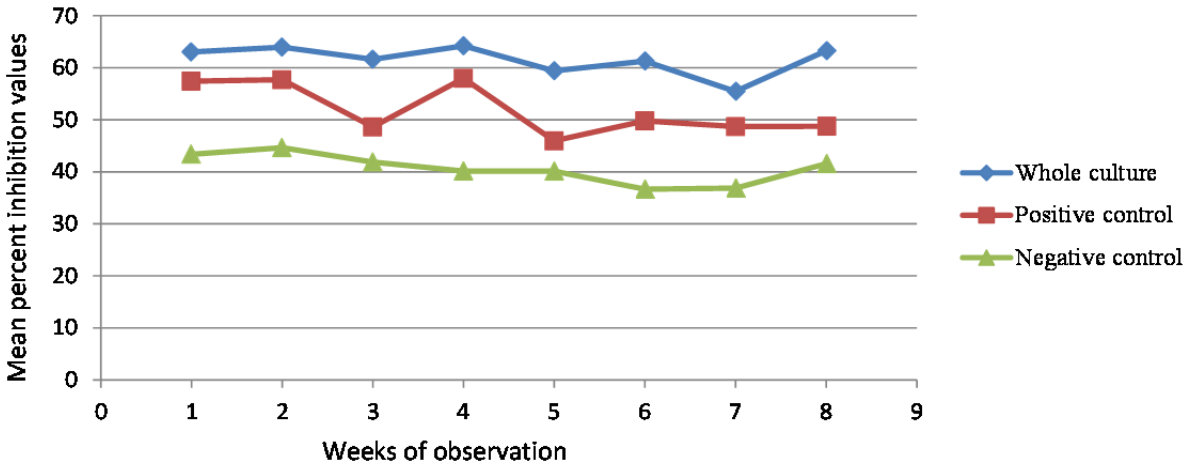

Figure 1. Line graph showing the highest value of mean percentage inhibition of seroconversion for whole culture trial vaccine relative to other groups.

inactivated CCPP vaccine at field level. However, it was impossible to compare the findings of the current study with study made elsewhere, as there was no previous works made on immunogenicity of inactivated whole culture CCPP vaccine and this is the first finding of its kind.

The mean percentage inhibition (PI) of sera of the three groups was also compared to evaluate the strength of sero-conversion during the experimental period. The result indicated that mean PI of whole culture was the highest (61.52±2.92), and the mean PI of positive control (51.86±4.95) was greater than that of negative control (40.65±2.84) and the difference was statistically significant (P=0.000). The reason needs further investigation but it might be attributed to quantity and type of antigen found in the inactivated whole culture CCPP trial vaccine which is whole in its nature and nothing was subtracted by concentration procedure of the existing vaccine (Tesgera and Tefera, 2012).

In this trial, immunized animals were able to produce antibody during the first week of vaccination and continued producing antibodies variably during the trial which lasted eight weeks. It has been known that the CCPP vaccine protect goats for one year (OIE, 2014).

In conclusion the newly designed inactivated whole culture CCPP trial vaccine is equally safe and immunogenic as the existing inactivated CCPP vaccine produced by concentration technique. But as the production procedure of the trial vaccine is easier, requires less time and is not capital investment intensive, the institute can shift its production strategy to the new methodology even though the correlation between this sero-conversion and field protection study remained to be done. Additionally the strength of antibody titer (as was seen by mean percentage inhibition) of whole culture trial vaccine was higher than that the vaccine used as positive control based on serological test(c-ELISA). So this inactivated trial whole culture CCPP vaccine could provide greater protection of goats than those vaccinated by existing vaccine but this needs further investigation by challenge study in the future.

Conflicts of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Molecular identification and characterization of *Salmonella* species isolated from poultry value chains of Gazipur and Tangail districts of Bangladesh

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The aim of this study was to identify and characterize *Salmonella* species isolated from poultry value chains of Gazipur and Tangail districts of Bangladesh during the period of October 2015 to May 2016. For this purpose a total of 153 samples (35 chick meconium, 49 cloacal swab, 30 poultry carcass, 14 feed, 16 water, 7 transport swab, 2 floor swab) were collected and were subjected to various cultural, biochemical techniques and polymerase chain reaction (PCR). Furthermore, the isolated *Salmonella* species were characterized by antimicrobial susceptibility testing. Among the samples, 23.53% (n=36) were found to be associated with *Salmonella* species. The *Salmonella* species were identified by observing black centered colonies on XLD agar, positive to MR test and negative to VP and Indole test. All isolates of *Salmonella* species were positive to 16s rRNA gene based PCR (574 bp). Serogrouping of *Salmonella* species were performed by slide agglutination test using commercial *Salmonella* specific polyvalent O (A-I) antisera, *Salmonella* O group B (Factor O: 4, 5, 27) antisera and *Salmonella* O group D (Factor O: 9, 46) antisera. Among the 36 isolates, 30.56% (n=11) belonged to serogroup B and rest of the isolates 69.44% (n=25) to serogroup D. The most prevalent serogroup identified in this study was serogroup D. The isolated *Salmonella* species were subjected to antimicrobial susceptibility testing with the aid of disk diffusion method using 8 antimicrobial agents. All isolates of *Salmonella* species were susceptible to ciprofloxacin, norfloxacin, streptomycin and gentamicin. Out of 36 isolates 100% *Salmonella* species were resistant to erythromycin and tetracycline. The findings of this study revealed the presence of multidrug resistant *Salmonella* species in poultry value chains of Gazipur and Tangail districts of Bangladesh that posseses a serious threat to public and poultry health. To the best of our knowledge, this is the first report on the prevalence, serogrouping and antimicrobial resistance patterns of *Salmonella* species from poultry value chains of selected districts in Bangladesh.

Key words: Isolation, identification, *Salmonella* species, poultry value chains, serogrouping, antibiogram study.

INTRODUCTION

Bangladesh is an agriculture based country. Poultry rearing is considered superior to the others in agricultural sector because of an almost assured and quick return in a relatively short period of time (Saleh et al., 2003).

Poultry industry which has started during 1980_s is an excellent agribusiness (Haque, 2001). Over the last decades surprising development in the poultry sector has been occurred (Rahman, 2003). It has become a vital sector for its employment generation, creating additional income and improving the nutritional level of the country. This sector provides fulltime employment to about 20% and partial employment to about 50% of the rural people (Alam et al., 2003). Development of poultry sector in Bangladesh is being hampered by a number of factors, of which the diseases are considered as the major factor causing 30% mortality of chicken per year (Das et al., 2005). Intestinal bacteria play an important role on health through their effects on gut morphology, nutrition, pathogenesis of intestinal diseases and immune responses (Mead, 2000).

Among the bacterial diseases, salmonellosis has been considered one of the most important infectious disease in both humans and animals (Keusch, 2002). Motile *Salmonellae* (paratyphoid group) infections cause salmonellosis in chickens and have zoonotic significance (Kabir, 2010). Salmonellosis is major problems in the poultry industry in Bangladesh (Haider et al., 2008). *Salmonella* infection is one of the major constraints of poultry farming that hindered its development in Bangladesh (Kamaruddin and Giasuddin, 2003; Das et al., 2005). It causes a variety of acute and chronic diseases of poultry in Bangladesh (Bhattacharjee et al., 1996). Chicks can be infected with *Salmonella* species by vertical transmission through infected parents or by horizontal transmission through hatcheries, sexing in contaminated hatcheries, cloacal infection and transportation of equipment and feed (Opitz et al., 1993). There are >2500 *Salmonella* serovars distributed throughout the world (L Plym and Wierup, 2006).

Several studies were carried out about the detection of *Salmonella* species in poultry in Bangladesh (Islam et al., 2016; Parvej et al., 2016; Al-salauddin et al., 2015; Jahan et al., 2013). Al-salauddin et al. (2015) recently conducted a study to isolate and identify the *Salmonella* species present in broiler meat. From 18 dressed broiler meat samples 31.66% *Salmonella* species were isolated. 55% *Salmonella* species were also isolated from 80 cloacal swab samples by Islam *et al*. (2016). 45% (n=27) bacterial isolates out of 60 samples were identified as *Salmonella* species from dressing water and environmental swabs by Jahan et al. (2013). This indicated a high rate of *Salmonella* contamination in poultry and live bird markets of Bangladesh, therefore salmonellosis status of a farm needs to be determined for its proper control and management (Ahmed et al., 2009). But no work has been done yet in Bangladesh to identify the *Salmonella* species from different phases of poultry value chains (hatchery → farm → transport → live bird markets) at a time. Therefore, the present study was designed to isolate and identify *Salmonella* species as well as serogrouping the isolated *Salmonella* species.

MATERIALS AND METHODS

Sample collection

A total of 153 samples (Table 1) were collected from poultry value chains of three different upazilas of Gazipur and Tangail districts of Bangladesh. During the collection of samples precautionary measures were taken to avoid contamination and ice boxes were used to maintain cool chain. Then the collected samples were brought to the Bacteriology Laboratory of the Department of Microbiology and Hygiene, BAU, Mymensingh for isolation, identification, serogrouping and antimicrobial susceptibility testing of *Salmonella* species.

Cultural characterization and isolation of *Salmonella* species

The samples were then cultured under aerobic conditions at 37ºC for 24 h using XLD agar. The growing colonies of *Salmonella* species were characterized morphologically using Gram's stain according to the method described by Merchant and Packer (1967) and motility test with hanging drop slide (Cowan, 1974).

Identification of *Salmonella* species by biochemical tests

Biochemical characterizations of the *Salmonella* isolates were performed with Sugar fermentation test, Methyl Red test (MR) and Voges-Proskauer test (V-P) (Cheesbrough, 1985).

Preparation of DNA templates

DNA template was prepared by boiling method described by Queipo-Ortuno et al. (2007). 250 µl distilled water was taken into Eppendorf tube and a pure *Salmonella* colony was picked up and mixed with the distilled water. The tubes then transferred to boiling water and boiled for 10 min then immediately transferred to ice for cold shock about 10 min and then centrifuged at 10,000 rpm for 10 min. Supernatant were collected and used as DNA template during PCR.

16S rRNA gene based PCR for identification of the genus *Salmonella*

The PCR analyses, reaction was carried out in a final volume of 20 µl containing 10 µl of master mixture (Promega, USA), 1 µl of each primer (forward and reverse/16s rRNA), 3 µl of DNA and 5 µl of deionized water. The amplification was carried out as follows:

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Table 1. Summary of collected samples from poultry value chains of Gazipur and Tangail districts of Bangladesh.

Type of samples	No. of collected samples	
	Gazipur district	Tangail district
Chick meconium	25	10
Cloacal swab	35	14
Whole Carcass	22	08
Feed	10	04
Water	12	04
Transport swab	05	02
Floor swab	02	-
Total	**111**	**42**

Initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 50°C for 30 s and extension at 72°C for 30 s. The final extension was conducted at 72°C for 5 min. After amplification, the samples were stored at 4°C. PCR products were analyzed by 1.5% agarose (Invitrogen, USA) gel electrophoresis and the bands were visualized with UV light after staining with ethidium bromide (0.5 µg/ml) for 10 min in a dark place. Bands were visualized and images were captured on a UV transilluminator (Biometra, Germany).

Serogrouping of *Salmonella* by O-antigen test

Salmonella agglutinating antiserum poly "O" and poly "A-I" (S & E Reagents Lab, Bangkok, Thailand) was used to perform the serotyping of the isolated Salmonella species. One drop of normal saline (0.85% NaCl) was added as control on a glass slide by the use of a wire. A loop full of culture from the Nutrient agar (NA) plate was transferred onto the glass slide and mixed with the drop of saline. Agglutination within 30 seconds indicated that it's rough strains. The strains can't be used for serotyping. Serotyping was continued with antisera if no agglutination was recorded. One drop of *Salmonella* agglutinating antisera (Poly A-I) was added on each test area on the slide. A loop full of culture from NA plate was added to each spot of antiserum. Mixed carefully the culture with the O-serum. The glass slide was rocked gently for one minute. Agglutination with the antisera indicated that the strain has an O-antigen. It was a screen procedure. Then tested with O group B and O group D. Some strain agglutinated with O group B (O: 4, 5, 27) and some strain agglutinated with O group D (O: 9, 46).

Antimicrobial susceptibility testing

All *Salmonella* species were tested against eight commonly used antibiotics (HiMedia, India) by the method of disk diffusion as described by Bauer et al. (1966). The zones of growth inhibition were compared with the zone size interpretative standards as described by Clinical and Laboratory Standard Institute (2011). *E. coli* ATCC 25922 was used as a quality control organism in this study. At least two separate experiments were performed for confirmation of all susceptibility data.

RESULTS AND DISCUSSION

A total of 153 samples (35 chick meconium, 49 cloacal swab, 30 poultry carcass, 14 feed, 16 water, 7 transport swab, 2 floor swab) were collected from poultry value chains of three different upazilas of Gazipur and Tangail districts of Bangladesh. Out of 153 samples, 147 samples were collected from hatcheries, farms and transports. Out of 147 samples, 30 (20.4%) samples have shown positive for *Salmonella* species (Table 2). A total of 6 samples were collected from 2 live bird markets of Gazipur district. All 6 (100%) samples were positive for *Salmonella* species in collected broiler meat, floor swab and water samples of Sreepur upazilla in Gazipur district as presented in Table 2.

For the cultural examination of *Salmonella* species several selective media such as XLD and SS agar were used which were also used by a number of researchers (Hyeon et al., 2011; Muktaruzzaman et al., 2010). In this study, *Salmonella* species were produced translucent, black smooth, small round colonies on SS agar and pink color colonies with black centre on XLD agar (Table 3). These findings were similar to the findings of other authors (Muktaruzzaman et al., 2010; Sujatha et al., 2003; Khan et al., 2005).

In Gram's staining, the morphology of the isolated *Salmonella* species exhibited Gram negative small rod arranged in single or paired (Table 3) which was supported by several researchers (Freeman, 1979; Buxton and Fraser, 1977; Merchant and Packer, 1967). Biochemical tests were performed for the identification of *Salmonella* species. In carbohydrate fermentation test, the isolates that fermented glucose, maltose and produced acid and gas but did not ferment lactose those indicated positive for *Salmonellae* as was stated by Buxton and Fraser (1977). All the isolates were found positive for MR test and negative to Indole test and V-P test (Table 4) which was supported by Cheesbrough (1985). Motility test was elementary basis for the detection of motile and non-motile *Salmonella* species. In motility test, 26 isolates were non motile and 10 isolates were motile (Table 3). That means the non-motile 26 isolates were either *Salmonella enterica* serotype *gallinarum or pullorum* and other isolates were motile species of *Salmonella* which was supported by Grimont et al. (2000).

In this study molecular identification was done by PCR, in which 16s rRNA gene was amplified for the detection of isolated *Salmonella* species and the results are shown in Figure 1. All conditions and results found in the PCR were similar by the findings of the several researchers (Ziemer and Steadham, 2003; Lin and Tsen, 1996).

Salmonella species were isolated from 30 apparently healthy broiler samples and positive isolates were 36.67% (n=11). In this study the incidence of *Salmonella* species in whole carcass was closely similar with the results reported by several researchers (Hossain et al., 2015; Ahmed et al., 2009; Zhao et al., 2001).

There were presence of 8.16% (n=4) *Salmonella*

Table 2. Summary of isolated *Salmonella* species from poultry value chains.

Placement (no. of farms)	No. of collected samples										*Salmonella* species No. of isolates (%)									
	Hatchery	Farm					Live bird market				Hatchery	Farm					Live bird market			
	CM	CS	F	W	WC	T	LWC	W	FS	Total	CM	CS	F	W	WC	T	LWC	W	FS	Isolates
Gazipur sadar, Gazipur (3)	15	21	6	6	12	3	1	1	1	66	2(13.3)	5(23.8)	1(16.6)	0(0)	4(33.3)	1(33.3)	1(100)	1(100)	1(100)	16(24.2)
Sreepur, Gazipur (2)	10	14	4	4	8	2	1	1	1	45	1(10)	2(14.2)	1(25)	0(0)	2(25)	0(0)	1(100)	1(100)	1(100)	9(20)
Total (Gazipur) (5)	25	35	10	10	20	5	2	2	2	111	3(12)	7(20)	2(20)	0(0)	6(30)	1(20)	2(100)	2(100)	2(100)	25(22.5)
Tangail sadar, Tangail (2)	10	14	4	4	8	2	-	-	-	42	1(10)	3(21.4)	2(25)	1(25)	3(37.5)	1(50)	-	-	-	11(26.19)
Gross total (7)	35	49	14	14	28	7	2	2	2	153	4(11.4)	10(20.4)	4(28.6)	1(7.1)	9(32.1)	2(28.6)	2(100)	2(100)	2(100)	36(23.53)

CM=Chick meconium, CS =Cloacal swabs, F=Feed, W=Water, WC=Whole carcass (broiler meat), T=Transport swabs, LWC=Live whole carcass and FS=Floor swabs.

Table 3. Results of cultural, morphological and motility characteristics of isolated *Salmonella* species.

Colony morphology		Staining characteristics	Motility
Xylose-Lysine Deoxycholate agar	Salmonella-Shigella agar	Pink short rod shaped, Gram negative bacteria arranged in single or paired.	+ve or -ve
Black centered colony.	Translucent, black smooth round colonies.		

"-Ve"=negative, "+Ve" = positive.

Table 4. Biochemical reaction patterns of *Salmonella* species.

Bacteria	Sugar fermentation properties					MR	VP	Indole
	Dextrose	Maltose	Sucrose	Lactose	Mannitol			
Salmonella species	AG	AG	-ve	-ve	AG	+ve	-ve	-ve

MR= Methyl red, VP = Voges-Proskauer reaction. , AG = Acid and Gas, "-Ve"=negative, "+Ve" = positive.

species in collected samples of chick meconium (49) from hatchery and this findings was very close to the findings of several researchers (Nasrin et al., 2012). These findings also indicate that vertical transmission of *Salmonella* species occurred to the chick that was similar to the findings reported by several researchers (Cason et al., 1994; Bailey et al., 1994).

In this study, 28.57% (n=7) of *Salmonella* species were positive in transport swabs samples (n=7). 28.57% (n=4) of *Salmonella* species were positive in feed samples (n=14). The prevalence of *Salmonella* species in feed sample was closely similar to the findings of Islam et al. (2014). There was also 18.75% (n=3) *Salmonella* species were present in water samples (n=16). The results of this study were closely related with the results of several authors (Saha et al., 2012; Samanta et al., 2014).

About 49 cloacal swab samples were collected for the research work. Among 49 cloacal swab

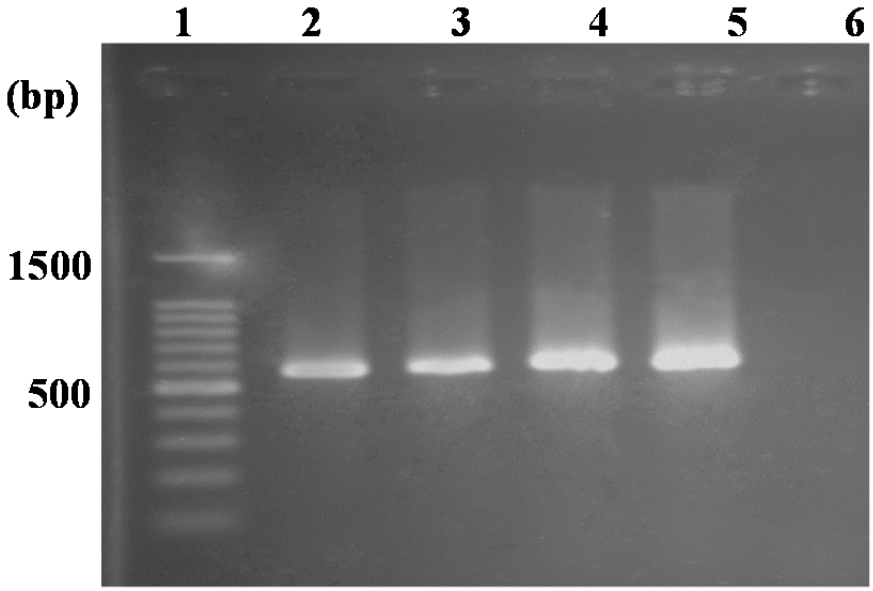

Figure 1. Detection of *Salmonella* species by 16s rRNA gene based PCR. Lane 1: 100 bp DNA ladder (Promega, USA); Lane 2, 3, 4, 5: DNA of *Salmonella;* Lane 6: Negative control.

Table 5. Serogrouping of *Salmonella* species.

Isolates	No. (%) of *Salmonella* species		
Salmonella species (n=36)	Poly A-I	Group B (O:4,5,27)	Group D (O:9,46)
	36 (100%)	11 (30.56%)	25 (69.44%)

samples 20.41% (n=10) samples were positive for *Salmonella* species. In between two districts higher percentage was observed 21.4% (n=3) in Tangail district of Bangladesh. The results of this study were in close relation with the results of several researchers (Islam et al., 2016; Parvej et al., 2016; Sarker et al., 2012). Cloacal swabs have been used to provide evidence of persistent intestinal colonization by *Salmonella* in individual birds reported by Gast et al. (1997). Among the 153 collected samples from two different districts, the total *Salmonella* species were isolated as 23.53% (n=36). The isolated *Salmonella* species in Gazipur district was 22.52% (n=25) and in Tangail district was 26.19% (n=11). Results of this study were closely related with the results of several researchers (Al-Ferdous et al., 2013; Kabir, 2010).

From the collected 153 samples, 36 *Salmonella* species were isolated. Among the 36 isolates, 30.56% (n=11) belonged to serogroup B and 69.44% (n=25) isolates belonged to serogroup D as presented in Table 5. The most prevalent serogroup identified in this study was serogroup D. The results of serogrouping correlated with the results of motility test where 26 isolates were non motile and 10 isolates were motile. These findings were in agreement with the result reported by several researchers (Mahmud et al., 2011; Arroyo and Arroyo, 1995).

Antimicrobial susceptibility analysis is presented in Tables 6 and 7. In this study it was revealed that *Salmonella* species were sensitive to ciprofloxacin, gentamicin and norfloxacin. This result was supported by a number of researchers (Jahan et al., 2013; Al-Ferdous et al., 2013; Khan et al., 2005). Out of the 36 *Salmonella* isolates 100% (n=36) were resistant to tetracycline which was similar to the report of (Lu et al., 2011). 13 (36.11%) were resistant to 2 agents E-TE. 6 (16.67%) were resistant to 3 agents E- AMX-TE. 9 (25%) were also resistant to 3 agents E-AZM-TE. Another 8 (22.22%) were resistant to 4 agents AMX-AZM-E-TE. Similar studies were also observed by several researchers (Al-Ferdous et al., 2013; Jahan et al., 2013; De et al., 2012; Hyeon et al., 2011; Khan et al., 2005). Resistant profile of *Salmonella* species were recorded some multi-drug resistant *Salmonella* species, which was similar to the result of some researchers (Al-Ferdous et al., 2013;

Table 6. Antimicrobial susceptibility pattern of *Salmonella* species by disk diffusion method.

Antimicrobial agents	No. (%) of *Salmonella* species		
	S	I	R
Amoxicillin	17 (47.22%)	5 (13.89%)	14 (38.89%)
Azithromycin	7 (19.44%)	12 (33.33%)	17 (47.22%)
Ciprofloxacin	36 (100%)	0 (0%)	0 (0%)
Erythromycin	0 (0.0%)	0 (0%)	36 (100%)
Gentamicin	36 (100%)	0 (0%)	0 (0.0%)
Norfloxacin	36 (100%)	0 (0%)	0 (0.0%)
Streptomycin	36 (100%)	0 (0%)	0 (0%)
Tetracycline	0 (0.0%)	0 (0%)	36 (100%)

S= Susceptible; I= Intermediate; R= Resistance

Table 7. Antimicrobial resistance profiles of *Salmonella* species.

Isolates	Resistance profiles	No. of isolates (%)
Salmonella species (n=36)	No resistance demonstrated	–
	Resistant to 2 agent (E-TE)	13 (36.11%)
	Resistant to 3 agents (E- AMX-TE)	6 (16.67%)
	Resistant to 3 agents (E-AZM-TE)	9 (25%)
	Resistant to 4 agents (AMX-AZM-E--TE)	8 (22.22%)
	Total resistant isolates	**36 (100%)**

AMX=Amoxicillin, AZM=Azithromycin, E=Erythromycin, GEN=Gentamicin, ClP=Ciprofloxacin, NOR=Norfloxacin, TE=Tetracycline, S=Streptomycin.

Jahan et al., 2013).

Conclusions

The findings of this study revealed the presence of multidrug resistant *Salmonella* species in poultry value chains of Gazipur and Tangail districts of Bangladesh that poses a serious threat to public and poultry health. Nevertheless, more studies are needed to clearly understand the genomic diversity in *Salmonella* species as well as molecular mechanisms for the development of antimicrobial resistance.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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