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Full Length Research Paper

Use of novel specific primers targeted to *pheS* and *tuf* gene for species and subspecies identification and differentiation of the *Bacillus subtilis* subsp. *subtilis*, *Bacillus amyloliquefaciens* and *Bacillus licheniformis*

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To clearly delimit the members of the *Bacillus subtilis* group (BSG), is difficult using common phenotypic and genotypic methods. This study described the use of *pheS* and *tuf* gene as targets for interspecies discrimination within the BSG, and also to develop specific PCR and SNP primers for species and subspecies identification and differentiation. The average sequence similarity values of the *pheS* and *tuf* gene among type strains were 85.1 and 94.7%, respectively, and all members of the BSG could be clearly distinguished based on phylogenetic analyses of *pheS* gene sequence. In addition, the specific primers were designed according to *pheS* and *tuf* gene sequence. The primers were shown to specifically identify *B. subtilis* subsp. *subtilis*, *B. amyloliquefaciens* and *Bacillus licheniformis*, and clearly differentiate the subspecies of *B. amyloliquefaciens* using specific-PCR, combined with two-plex minisequencing method. In conclusion, we have successfully established a comparative sequence analysis and rapid molecular diagnosis techniques for determination of interspecies within the BSG.

Key words: *Bacillus subtilis* group (BSG), species and subspecies discrimination, comparative sequence analysis, specific Pcr, two-plex minisequencing.

INTRODUCTION

The *Bacillus subtilis* group (BSG) contains more than 10 closely related taxa (Dunlap et al., 2015), and have some beneficial effects of BSG members as reported, like the production of enzymes, antibiotics, vitamins and fermented foods, which are commonly applied as animal feeds additives (Sorokulova et al., 2013; Kubo et al., 2011). Many studies have demonstrated that BSG strains have beneficial effects on production performance in domestic

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animals (Alexopoulos et al., 2004; Kritas et al., 2006; Knap et al., 2010; Ahmed et al., 2014). However, the species and subspecies determination of these phylogenetically related bacteria has long been problematic.

DNA-DNA hybridization (DDH) is the gold standard for bacterial species delineation (Stackebrandt et al., 2002), but this method is time-consuming, labor intensive, costly and difficult to use routinely in laboratories. To date, a comparative analysis on the 16S rDNA is a commonly used genotypic method for bacterial identification, and the strains that show at least 98.7% sequence similarity between the 16S rDNA are recognized to the same species (Stackebrandt and Ebers, 2006).

Unfortunately, poor discrimination has been observed in BSG, due to the high degree of similarity (reaching 99-100%) of the 16S rRNA gene sequences (Wang et al., 2007). In contrast, DNA sequences of housekeeping genes with a higher resolution seem to be more effective than the 16S rDNA and may act as an alternative to DDH, for species determination (Guo et al., 2012). The *tuf* gene encodes the elongation factor Tu associated with protein biosynthesis, which facilitates the aminoacyl-tRNA to the ribosomes during the translation process. Moreover, *tuf* is universally distributed, and the various copy numbers (one to three) per bacterial genome have been found (Ke et al., 2000). The *tuf* gene is ideally suited for inferring phylogenetic relationships between bacteria (Chavagnat et al., 2004; Picard et al., 2004). Phenylalanyl-tRNA synthase gene (*pheS*) has also been proposed as a useful molecular marker in the closely related species complex (Naser et al., 2005; Naser et al., 2007). In this study, we determined the utility of *pheS* and *tuf* genes sequences for species and subspecies discrimination in BSG, and as targets to develop specific primers for identification and differentiation.

MATERIALS AND METHODS

Bacillus strains and culture conditions

All BSG type strains and isolates were obtained from Bioresource Collection and Research Center (BCRC) and are listed in Table 1. *Bacillus* strains were incubated aerobically on Nutrient agar (NA, Difco) for 24 h at 30°C.

Genomic DNA preparation and design of degenerate primers

The chromosomal DNA was extracted using the DNeasy Kit (Qiagen, Valencia, CA, USA), and the DNA concentration and purity were measured using an absorbance ratio of 260/280 nm and checked by agarose gel electrophoresis. By comparison with the *pheS* and *tuf* genes from the whole genome sequence in BSG species (Accession no: CP002905, AL009126, CP002207, FN597644, CP000560, CP000002), the degenerate primers, pheS-21F: 5'-CAYCCNGCHCGYGAYATGC-3' and BasbpheS-416R: 5'-ARYACRTTCGGRTGAACCAT-3', and Basbtuf-F1: 5'-CAAACTCGTGAGCACATYCT-3' and Basbtuf-R1: 5'-CGTCAGTTGTACGGAARTAG-3', were designed and targeted to the most conserved region of the gene.

Target gene amplification and DNA sequencing

The partial fragments of *pheS* and *tuf* genes of BSG related strains were amplified and sequenced using consensus degenerate primers. The thermal protocol was carried out under the following conditions: 5 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C; and 7 min at 72°C. The resulting amplicons were purified using the QIAquick PCR purification Kit (Qiagen Inc., Valencia, CA, USA) and sequenced with the BigDye Terminator v3.1 cycle-sequencing Kit on the 3730 DNA sequencer (Applied Biosystems and Hitachi, Foster City, CA, USA).

Intespecific bioinformatic analysis

The *pheS* and *tuf* gene sequences of all strains were aligned using the Clustal X program (version 1.8). The DNA sequence similarities were calculated using the MatGAT (version 2.02) (Campanella et al., 2003). Phylogenetic tree was performed with the PHYLIP (version 3.63) package, using the neighbour-joining method (Felsenstein, 2004; Kimura, 1980; Saitou and Nei, 1987).

Species and subspecies-specific primers design and PCR identification

The PCR oligonucleotide primers is specific for *B. subtilis* subsp. *subtilis*, *B. amyloliquefaciens* and *Bacillus licheniformis* were designed based on the *pheS* and *tuf* gene sequences, and all reference strains were used for specific PCR testing. The thermal protocol was carried out under the following conditions: 5 min at 94°C; 25 cycles of 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C; and 7 min at 72°C.

Subpecies-specific SNaPshot mini-sequencing assay

The SNP primers specific for *B. amyloliquefaciens* subspecies were designed based on the *pheS* gene sequences. The mini-sequencing protocol and final data analysis were followed as previously described (Huang et al., 2014).

RESULTS AND DISCUSSION

In this study, partial *pheS* and *tuf* gene fragments (approximately 410 bp and 750 bp) were successfully amplified from all BSG strains and were used for sequencing, by using degenerate primers. The average sequence similarity values for *pheS* and *tuf* genes among BSG type strains were mean 85.1 and 94.7%, respectively, which exhibit greater variation than 16S rDNA sequence (98.9%). The topology of the *pheS* tree showed all BSG members that could be discriminated (Figure 1). Nowadays, several molecular targets have been exploited to differentiate BSG members. The three targets (*gyrA*, *gyrB* and *phoR*) showed good resolution with a high discrimination power, and the average

Table 1. Strains used in this study and their detection using each primer pairs.

Bacillus spp.	BCRC strains	Species and subspeices-specific PCR assays			
		Bamy[a]	Blic[b]	Blic[c]	Bsub[d]
Bacillus licheniformis	11702^T, 11718, 11594, 11958, 11978, 14353, 10287, 10259	−	+	+	−
Bacillus sonorensis	17416^T, 17532	−	−	−	−
Bacillus subtilis subsp. *subtilis*	10255^T, 12144, 10872, 10058, 11602, 14717, 17441, 17435, 17436, 17437, 17438, 17439, 17440, 17442, 17443, 12141, 17890, 10613, 10614, 10615, 10616, 10617, 11703, 12142, 14645, 14638, 14639, 14640, 14199, 10258	−	−	−	+
Bacillus subtilis subsp. *inaquosorum*	17984^T, 17975, 17976, 17977, 17978, 17979, 14192, 80075	−	−	−	−
Bacillus subtilis subsp. *spizizenii*	17366^T, 10447, 14643, 14644, 80045, 80046, 80047, 80076	−	−	−	−
Bacillus mojavensis	17124^T, 17501, 17502, 17531, 17653, 17654, 80138, 80139, 80140,	−	−	−	−
Bacillus amyloliquefaciens subsp. *amyloliquefaciens*	11601^T, 11266, 17038, 14710, 14711, 12815, 11199, 10453, 14637	+	−	−	−
Bacillus amyloliquefaciens subsp. *plantarum*	80282^T, 80285, 80604, 80110, 11557, 14193, 17650, 17710	+	−	−	−
Bacillus atrophaeus	17123^T, 17530	−	−	−	−
Bacillus siamensis	80787^T	−	−	−	−
Bacillus vallismortis	17183^T, 80798, 80799	−	−	−	−
Bacillus tequilensis	17634^T	−	−	−	−

BCRC, Bioresource Collection and Research Center at Food Industry Research and Development Institute, Taiwan; +, PCR amplicons with each primer pair detected; −, PCR amplicons with each primer pair not detected. [a], species-specific-PCR amplification with primer spBamyphes-171F1 (5'-TCTCGTCGTSGACCGCAA-3')/353R1 (5'-GATCCAGCCTGTTTTTTTGC-3'); [b], species-specific-PCR amplification with primer spBlichphes-177F1 (5'-CGTTGACCGTGATATCAGCAT-3')/304R1 (5'-GCAGGAAACATCGACTTC-3'). [c], species-specific-PCR amplification with primer spBlichtuf-118F1 (5'-CTTTCTGAGTATGAGTTCC-3')/210R1 (5'-CCKCCATCAGTTCRAAGATT-3'). [d], subspecies-specific-PCR amplification with primer spBsubtuf-190F1 (5'-GACGCTGAGTGGGAAGCTAA-3')/363R1 (5'-ATGATTTCAACTTCGTCACCG-3').

sequence similarity values of these targets were 83.7, 83.3 and 77.9%, respectively (Chun and Bae, 2000; Guo et al., 2012; Wang et al., 2007). Therefore, the *pheS* gene can be as an additional phylogenetic marker for differentiating among the BSG.

On the other hand, all DNA sequences were submitted to GenBank (accession number: KX987658-KX987837), and this accumulated sequence data could be applied to design specific primers for direct identification of particular microbials (Krawczyk et al., 2002). The species-specific primer has been established for *B. subtilis* based on Endo-beta 1,4-glucanase gene and *ytcP* (encoding a hypothetical protein similar to a ABC-type transporter) gene (Ashe et al., 2014; Kwon et al., 2009). To the best of our knowledge, there were no such studies, in the identification of *B. subtilis* at subspecies level. Four primer pairs were designed based on multiple alignments of the *pheS* and *tuf* sequences, and these primers successfully generated a single species and subspecies-specific band (202 bp, 145 bp, 112 bp and 194 bp) when used in PCR reactions with *B. amyloliquefaciens*, *B. licheniformis* and *B. subtilis* subsp. *subtilis* DNA (data not shown).

Moreover, the amplified fragments were sequenced, and the results demonstrated that the sequence agreed with what were expected. Annealing temperatures and additional PCR amplification cycles may influence PCR specificity (Krawczyk et al., 2002). In the present study, the most appropriate conditions for our primer pairs were an annealing temperature of 65°C and 25 cycles of PCR amplification. The specificity of these primer pairs were tested against the

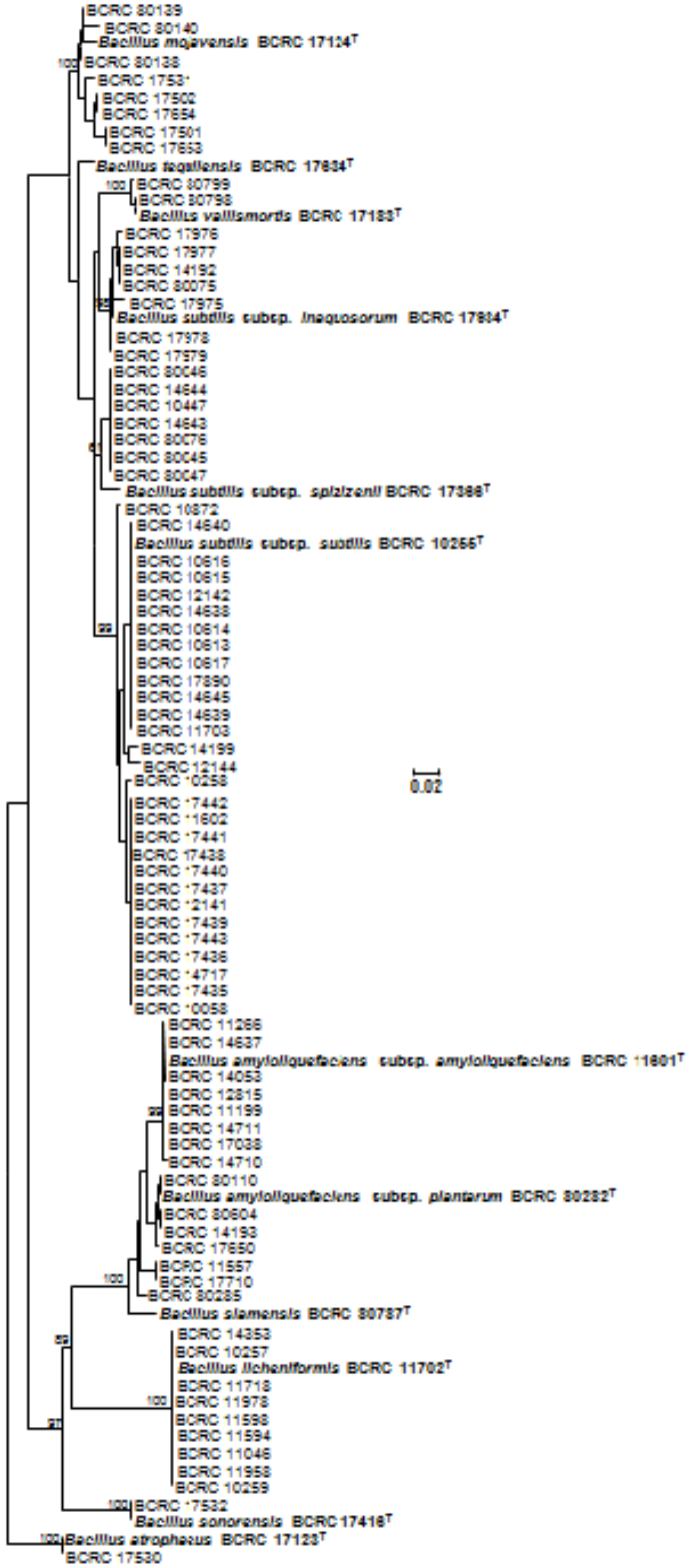

Figure 1. Phylogenetic tree of the BSG strains based on *pheS* gene sequences. The tree was constructed using the neighbor-joining method. Only bootstrap percentages above 60% are indicated (based on 1,000 replications). The scale bar represents 0.02% sequence divergence.

Table 2. Single nucleotide polymorphisms in *pheS* gene sequences of the subspecies within the *B. amyloliquefaciens* strains.

***B. amyloliquefaciens* subspecies**	**Sequence at the indicated position:**	
	231	**306**
amyloliquefaciens (no.=9)	G	A
plantarum (no.=8)	C	G

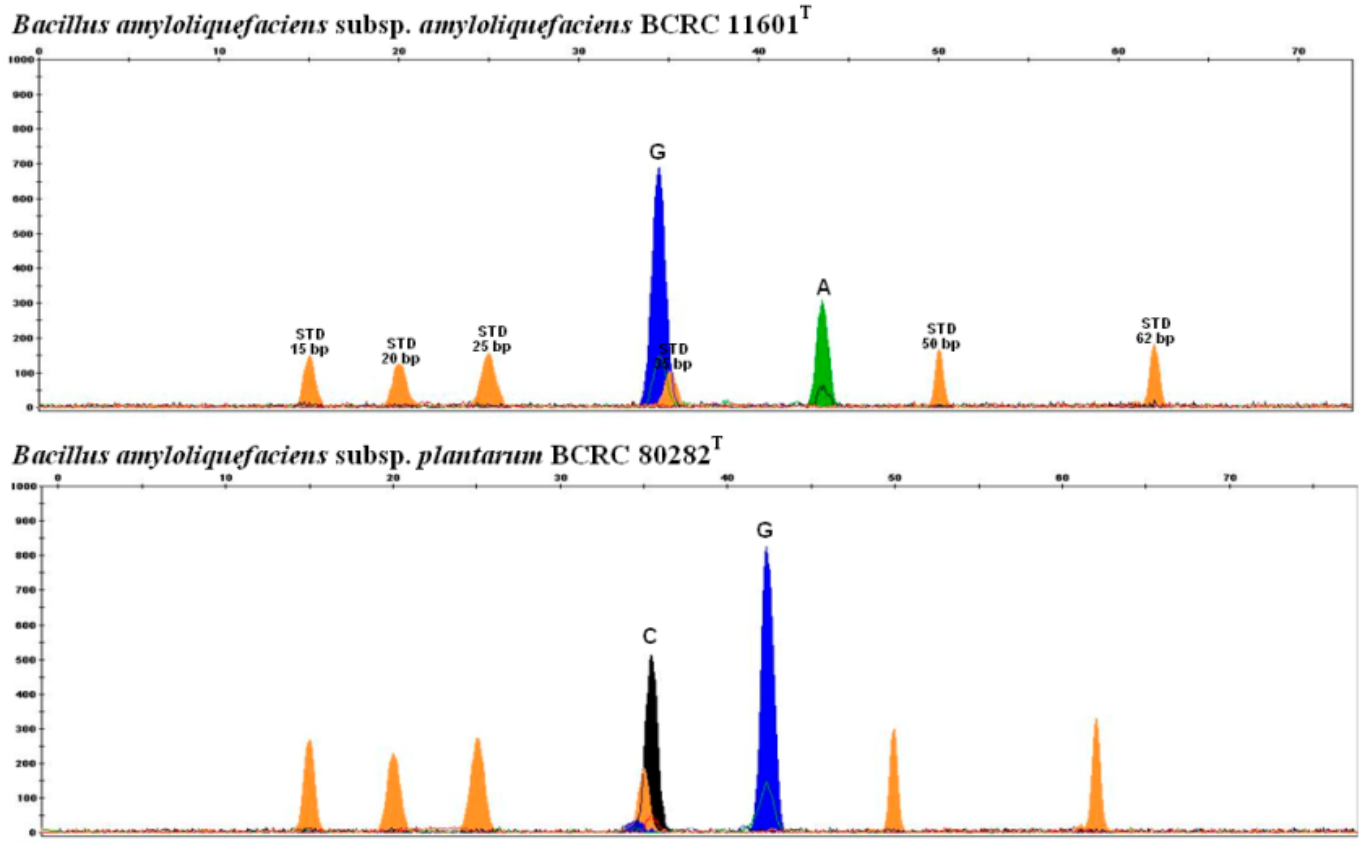

Figure 2. Electropherograms obtained from *B. amyloliquefaciens* strains by mini-sequencing assay. The *X*-axis represents the size of the mini-sequencing products (nucleotides); the *Y*-axis represents relative fluorescence units (RFUs). STD: GS120 LIZ size standard.

organisms indicated in Table 1.

In addition, five strains of *B. subtilis* subsp. *subtilis* were isolated from probiotic and feed additive samples, which were identified based on the DNA sequencing and specific-PCR method (data not shown).

Although *B. amyloliquefaciens* strains could be preliminary identified using species-specific PCR, but this method was not able to provide an accurate discrimination at the subspecies level. Afterward, a mini-sequencing assay was applied. The SNP specific primers spBamyphes-231f1 (5'-GCACGCTTGAATTGGTYGC-3') and spBamyphes-306f1 (5'-GACTGACTGACTCRTTCACAGAGCCTTCTGTCGA-3') were designed to anneal immediately, adjacent to the nucleotide at two subspecies-specific SNPs found at positions 231 and 306 in the alignment of all *B. amyloliquefaciens pheS* gene sequences (Table 2). Following the above, *B. amyloliquefaciens* species-specific amplicons containing two diagnosis sites were purified and subjected to a duplex mini-sequencing reaction. The results showed the presence of two peaks of the expected color and position in all samples (Figure 2; Table 1). Compared to other genotypic methods for strain differentiation of *Bacillus* spp. such as repetitive element palindromic PCR (rep-PCR), PCR restriction fragment length polymorphism (RFLP) and DNA

sequencing (Banyko and Vyletelova, 2009; Freitas et al., 2008; Jeyaram et al., 2011; Shaver et al., 2002), the mini-sequencing method is more direct and rapid due to its determined exact single nucleotide polymorphism at diagnosis sites.

Conclusion

All members of BSG can be clearly discriminated by housekeeping gene sequencing, and the developed specific primers can be successfully applied to quickly and accurately identify the *B. subtilis* subsp. *subtilis*, *B. amyloliquefaciens* subspecies and *B. licheniformis* using specific PCR combined with mini-sequencing assay.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Multidrug resistant *Campylobacter* in faecal and carcasses of commercially produced poultry

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Poultry meat and products are major transmission routes of human campylobacteriosis. The aim of this study was to determine the numbers and antibiogram profile of *Campylobacter* isolates from slaughtered broiler and layer birds. One hundred and sixty caecal and one hundred and thirty two carcasses were randomly sampled at the Kejetia poultry slaughter, isolated on charcoal-cefoperazone-deoxycholate agar (CCDA) and confirmed by API CAMPY and their resistance profiles assessed by Kirby-Bauer disk diffusion. Prevalence was 22.5 and 21.9% in the faecal and carcasses, respectively with no significant differences. Species identified among faecal isolates were *Campylobacter jejuni* (42%), *Campylobacter coli* (28%), *Campylobacter lari* (22%) and *Campylobacter hyo-intestinalis* (8%) while 79% *C. jejuni,* 14% *C. coli*, 4% *C. jejuni sub sp. doylei*, and 3% *C. lari* were obtained from the carcasses. Resistance to the β-lactams ranged from 75 to 100%, 41 to 86% to the quinolones, 14 to 36% to the aminoglycosides, 100% to erythromycin, 97 to 100% to tetracycline, 72 to 83% to chloramphenicol and 90 to 94% to trimethoprim sulfamethoxazole. All species were sensitive to imipenem, but 100% of isolates were multidrug resistant. Contamination of carcasses with multidrug resistant strains of *Campylobacter* is a threat to handlers and consumers and of major public health issue.

Key words: Multidrug resistance, faeces, carcass, poultry, Kumasi, Ghana.

INTRODUCTION

Campylobacter, a key zoonotic pathogen is among the most commonly reported agent of enteritis in humans worldwide, with *Campylobacter jejuni* and *Campylobacter coli* accounting for almost 90% of human infections (Scallan et al., 2011). *C. jejuni* is particularly adapted to poultry, being the largest reservoir of the pathogenic species (Rizal et al., 2010). *Campylobacter* is mainly haboured in the intestinal tract of warm-blooded animals

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and birds with the caeca, colon and cloaca of birds as the main sites of colonization (Humphrey et al., 2007). Consumption and handling of raw or undercooked poultry has most often been implicated in human infections (Denis et al., 2011). *Campylobacter* enteritis is usually self-limiting warranting no antimicrobial therapy; but in severe enteritis with complications and in cases of the immune-compromised, antibiotics are necessary in which case the fluoroquinolones and erythromycin are the drugs of choice (Luangtongkum et al., 2009). Moreover increasing resistance among *Campylobacter* has been reported from different geographical regions to the drugs of choice and other relevant antibiotics used in both human and veterinary medicine (Salihu et al., 2009; Luangtongkum et al., 2009; Tambur et al., 2009). Multidrug-resistant *C. jejuni* and *C. coli* have been reported from food animals and retail meats, including poultry (Ge et al., 2003; Gebreyes et al., 2005).

The use and abuse of antimicrobial agents in veterinary medicine or as feed additives have been recognized to be a major determining factor in the growth and dissemination of resistance in most bacterial pathogens. The abuse of antibiotics in animals may cause an increase in resistance of their enteric flora. These resistant bacteria from animals can then be transferred to the human population through direct contact or as food products from animal sources (Fallon et al., 2003). Although prevalence of *Campylobacter* in poultry and poultry products is well documented globally, information on its occurrence and resistance levels in poultry is underreported in Ghana. Moreover, antibiotics are extensively used in commercial poultry production in Ghana due to myriad disease challenges faced by poultry farmers (Aning, 1995). Establishing the resultant effects of these drugs on the resistance levels in the gut flora and carcasses of these poultry birds is necessary. Therefore this study described the occurrence of *Campylobacter* in the gut content and carcasses of slaughtered birds and assessed the resistance levels of species to 13 relevant antibiotics.

MATERIALS AND METHODS

The study was undertaken at the Kejetia central market in Kumasi, Capital of Ashanti Region of Ghana. Kejetia market is the largest open air market in Ghana, and the second largest in Africa. Ninety (90) Poultry traders, who are mostly women purchase live birds from commercial poultry farms within and outside the Metropolis, keep them in holding pens from where they are sold live or slaughtered, processed and packaged on site.

Sample collection

With permission from the traders, whole intestines were obtained intact from slaughtered broiler and layer birds into sterile ziplock bags, kept on ice packs and immediately transported to the laboratory from April, 2013 to June, 2013. In the laboratory, the caeca were cut with sterile scissors (sterilized using a burning flame) from the remaining part of the intestines. Chicken carcasses were randomly swabbed and inoculated into sterile Amies transport medium (eswab sticks, Copan, Italy) and returned on ice packs to the laboratory from May, 2013 to March, 2014. An average of 10 chicken swab and 7 faecal samples were, respectively obtained monthly and weekly for the study period.

Sample processing, isolation and identification

Caecal contents were emptied aseptically into sterile bijou bottles while swab sticks together with the transport media were aseptically transferred into bijou bottles and subsequently pre- enriched with 5 ml of blood-free Campylobacter broth (Oxoid CM0963, Denmark) each and incubated overnight at 37°C. The overnight enrichment culture was sub cultured onto mCCDA agar (Oxoid CM0689, Denmark), incubated at 42°C for 48 h using CampyGen (Oxoid CN0025A) to generate microaerophilic condition (FDA BAM, 1998). Biochemical tests including Gram stain, oxidase and catalase were performed on colonies showing typical morphology of *Campylobacter* spp. Isolates which were small, curved Gram negative bacilli, catalase and oxidase positive, were further subjected to standard phenotypic tests using API CAMPY (bioMerieux, France) to identify to species level.

Antibiotic susceptibility testing

The Kirby-Bauer disk diffusion method was carried out using Mueller-Hinton agar (Liofilchem-Italy) supplemented with 5% sheep blood. Plates were inoculated with 0.5 Mcfarland suspension and incubated microaerophilically at 48°C for 24 h (Clinical and Laboratory Standards Institute, CLSI, 2006). Drugs analysed were sourced from ROSCO (Denmark) and included: Ampicillin (10 µg/disc), chloramphenicol (30 µg/disc), ciprofloxacin (5 µg/disc), kanamycin (30 µg/disc), erythromycin (15 µg/disc), gentamicin (10 µg/disc), nalidixic acid (30 µg/disc), tetracycline (30 µg/disc), cephalexin (30 µg/disc), trimethoprim sulfamethoxazole (25 µg/disc), norfloxacin (10 µg/disc), cefotaxime (30 µg/disc) and imipenem (10 µg/disc). The recorded inhibition zones were interpreted according to EUCAST-CLSI (2013) breakpoints for *Campylobacter*. Established breakpoints by EUCAST and CLSI (2013), for enterobacteriaceae were used to interpret the results of norfloxacin, trimethoprim sulfamethoxazole, cefotaxime and kanamycin as CLSI breakpoints for these antibiotics are yet to be established for *Campylobacter*. Quality control strains of *Escherichia coli* (ATCC® 25922 ™) and *Staphylococcus aureus* (ATCC® 25923 ™) were used.

Statistical analysis

Percentages were calculated for the descriptive analysis. Associations were determined using the Chi-square test at a significance level of < 0·05. Fisher's exact test was used for expected frequencies being less than 5. All statistical tests were two-tailed. Stata 14.0 software was used for statistical analysis.

RESULTS

Prevalence of *Campylobacter* species in poultry faeces and carcasses

Of the 160 faecal content examined from Broilers (46)

Table 1. Prevalence of *Campylobacter* spp. in faecal contents and carcasses of poultry.

Poultry	No. Isolates examined		No. isolates Identified		Chi-square, df
	Faecal	Carcass	Faecal	Carcass	P-value
Layers	114	112	17 (10.6)	15 (11.3)	0.01175,1
Broilers	46	20	19 (11.8)	14 (10.6)	0.914
Total	160	132	36 (22.5)	29 (21.9)	

Values in bracket indicate percentage

Table 2. Distribution of *Campylobacter* spp. among poultry faecal isolates.

Poultry	No. isolates Identified	*Campylobacter spp.* identified			
		C. jejuni	*C. coli*	*C. lari*	*C. hyointestinalis*
Layers	17	8 (47)	4 (23)	3 (18)	2 (12)
Broilers	19	7 (37)	6 (32)	5 (26)	1 (5)
Total	36	15 (41.6)	10 (27.7)	8 (22.2)	3 (8.3)

Values in bracket indicate percentage.

Table 3. Distribution of *Campylobacter* spp. among poultry carcass isolates.

Poultry	No. isolates identified	*Campylobacter* spp. identified			
		C. jejuni	*C. doylei*	*C. coli*	*C. lari*
Layers	15	13 (87)	0 (0)	1 (7)	1 (6)
Broilers	14	10 (72)	1 (7)	3 (21)	0 (0)
Total	29	23 (79.3)	1 (3.4)	4 (13.7)	1 (3.4)

Values in bracket indicate percentage.

Of the 160 faecal content examined from Broilers (46) and Layers (114); 36 (22.5%) were confirmed as *Campylobacter* spp. Of the 132 poultry carcasses processed, 29 (21.9%) were positive for *Campylobacter spp.* (Table 1). No significant difference were observed in the isolation rates from faecal and carcass samples (p= 0.914). Four main species and one subspecies of *Campylobacter* were isolated from the samples. *Campylobacter jejuni* and *C. coli* were the dominant species followed by *C. lari* with C. *jejuni* subsp. *doylei* being the least. Faecal samples accounted for 41.6% for *C. jejuni*, 27.7% for *C. coli*, 22.2% for *C. lari* and 8.3% for *C. hyointestinalis* (Table 2) and in their carcasses, 79.3% for *C. jejuni,* 13.7 % for *C. coli*, 3.4% for *C. lari* and 3.4% for *C. jejuni* subsp*. doylei* (Table 3).

Antibiotic resistance patterns of poultry isolates

Resistance among faecal isolates to erythromycin and Ampicillin was 100% each and to tetracycline, trimethoprim sulfamethoxazole (SXT) and chloramphenicol were 97, 94 and 72%, respectively. Against the cephalosporins resistance was 86% to cephalexin and 75% to cefotaxime. Resistance to the quinolones was 86% to ciprofloxacin, 78% to nalidixic acid and 44% to norfloxacin. Against the aminoglycosides resistance was 36% to kanamycin and 14% to gentamicin. No resistance (0%) was observed against imipenem (Table 4). Carcass isolates showed 100% resistance each to tetracycline, erythromycin and Ampicillin, 90 and 83%, respectively to trimethoprim sulfamethoxazole (SXT) and chloramphenicol. Against the cephalosporins resistance was 100% to cephalexin and 97% to cefotaxime. Resistance to the quinolones was 59% to ciprofloxacin, 55% to nalidixic acid and 41% to norfloxacin. Resistance to aminoglycosides was 24% to gentamicin and 21% to kanamycin. No (0%) resistance was observed against imipenem (Table 4).

Species specific resistance profile of poultry isolates

Campylobacter jejuni resistance ranged from 87.2 to100% to the β-lactams, 23.1 to 28.2% to the

Table 4. Antibiotic resistance and susceptibility patterns of *Campylobacter* species from poultry.

Antibiotic	Faecal n=36			Carcass n=29		
	% S	% I	% R	% S	% I	% R
Nalidixic acid	22	NA	78	45	NA	55
Norfloxacin	31	25	44	45	14	41
Ciprofloxacin	6	8	86	14	27	59
Ampicillin	0	0	100	0	0	100
Cefotaxime	17	8	75	0	3	97
Cephalexin	14	NA	86	0	NA	100
Kanamycin	39	25	36	65	14	21
Gentamicin	78	8	14	62	14	24
Erythromycin	0	NA	100	0	NA	100
Tetracycline	3	0	97	0	0	100
Chloramphenicol	8	20	72	3	14	83
SXT	6	0	94	7	3	90
Imipenem	75	25	0	83	17	0

KEY: S=sensitive; I=intermediate; R=resistant; NA= intermediate not available.

aminoglycosides, 38.5 to 69.5% to the quinolones, and 100% each to erythromycin and tetracycline, 84.6% to chloramphenicol and 92.3% to trimethoprim sulfamethoxazole. *C. coli* strains showed resistance of 85.7 to 100% to the β-lactams, 0 to 28.6% to the aminoglycosides, 0 to 64.3% to the quinolones, 100% to erythromycin, 92.9% to tetracycline, 64.3% to chloramphenicol and 92.9% to trimethoprim sulfamethoxazole. Resistance among *C. lari* strains was 100% to all antibiotics with the exception of norfloxacin, chloramphenicol, trimethoprim sulfamethoxazole and aminoglycosides where resistance of 77.8, 55.6, 88.9 and 33.3% each was, respectively observed (Table 5). Differences in resistance rates among the different species to the various antibiotics was not statistically significant ($p > 0.05$), with the exception of norfloxacin and nalidixic acid ($p < 0.001$). Resistance to 3 or more antibiotics was defined as multidrug resistance (MDR) in this study and 100% was observed among the faecal and carcass isolates (Table 6).

DISCUSSION

Campylobacter contamination of commercially produced poultry birds slaughtered in Kejetia, a suburb of Kumasi, Ghana, was 22.5%, which was expected because *Campylobacter* are frequent colonizers of the intestinal tracts of birds especially poultry and falls within the reported global range of 10 to 90% (Jacob-Reitsma et al., 1994; Newell and Fearnley, 2003). This study recorded rate is however higher than the 14.1% earlier reported by Sackey et al. (2001) but lower than the 43.6% by Abraham et al. (1990) in Ghana. In South Africa, 47% has been reported in commercial and industrial broilers and 94% in industrial layers, 51.5% has been described in Nigeria, 63.8% in Cote d'Ivoire, 83.1% in Ireland and 87.2% in Poland (European Food Safety Authority, EFSA, 2010; Bester and Essack, 2012; Salihu et al., 2012; Bernadette et al., 2012; Wieczorek et al., 2012). Countries with low *Campylobacter* colonization rates in poultry has been attributed to limited small-scale poultry farms with high biosecurity levels which are measures lacking in our subregion (Johnsen et al., 2006).

Campylobacter spp. are common contaminants of poultry carcasses with prevalence of 20 to 100% established in fresh chicken from several countries (Dominguez et al., 2002; Jorgensen et al., 2002; Son et al., 2007). This study recorded contamination levels of 21.9% which is much lower than the 100% reported by Jozwiak et al. (2006) in Broiler chicken in Hungary. Similarly, 58.9, 69 and between 70.7 to 91.4% have been reported in Poland, Iran and Malaysia, respectively (Tang et al., 2009; Wieczorek et al., 2012; Bagherpour et al., 2014). The contamination of the poultry carcasses in this study could be as a result of the lack of a well-structured processing plant (facility), slaughtering in open air with inadequate rinsing and washing facilities and poor environmental hygiene.

Campylobacter jejuni was the dominant species; (42, 79%), followed by *C. coli* (28, 14%), from the faeces and carcasses, respectively. This finding affirms the dominance of *C. jejuni* in poultry and poultry products (Jorgensen et al., 2002; Son et al., 2007; Salihu et al., 2012). Nevertheless, the high recovery of the thermophilic *Campylobacters* in comparison with the non-thermophiles could be imputed to the selective nature of the CampyGen gas generating system which optimizes the growth of

Table 5. Species specific resistance profile of poultry isolates.

Antibiotic	*C. jejuni* n=39	*C. coli* n=14	*C. lari* n=9	P-value
	(%) resistance	(%) resistance	(%) resistance	
Nalidixic acid	15 (38.5)	1 (7.4)	9 (100)	<0.001
Norfloxacin	19 (48.7)	0 (0)	7 (77.8)	<0.001
Ciprofloxacin	27 (69.2)	9 (64.3)	9 (100)	0.128
Ampicillin	39 (100)	14 (100)	9 (100)	-
Cefotaxime	34 (87.2)	12 (85.7)	9 (100)	0.506
Cephalexin	39 (100)	12 (85.7)	9 (100)	0.029
Kanamycin	11 (28.2)	4 (28.6)	3 (33.3)	0.954
Gentamicin	9 (23.1)	0 (0)	3 (33.3)	0.089
Erythromycin	39 (100)	14 (100)	9 (100)	-
Tetracycline	39 (100)	13 (92.9)	9 (100)	0.175
Chloramphenicol	33 (84.6)	9 (64.3)	5 (55.6)	0.097
SXT	36 (92.3)	13 (92.9)	8 (88.9)	0.934

SXT=Trimethoprim sulfamethoxazole.

Table 6. Multidrug resistance among *Campylobacter* spp. from poultry.

Poultry			p-value	chi-square
Faecal	36	36(100.0)	1.0000	-
Carcass	29	29(100.0)		
Total	65	65(100.0)		

Multidrug resistance defined as resistance to 3 or more antibiotics

thermophilic *Campylobacters* but inhibits the growth of non-thermophiles by not producing hydrogen enriched atmosphere, which is required by the non-thermophilic *Campylobacters* (Workman et al., 2005).

Resistance was commonly observed against Ampicillin, tetracycline, chloramphenicol, erythromycin, trimethoprim sulfamethoxazole, cefotaxime, ciprofloxacin and nalidixic acid with moderate and no resistance against gentamicin and imipenem, respectively. The resistance levels in our study were comparable to work from different countries (Sukhapesna et al., 2005; Tang et al., 2009; Usha et al., 2010; Mansouri-najand et al., 2012; Kovalenko et al., 2014) although lower resistance have also been reported by Fallon et al. (2003). High resistance was generally observed among *C. jejuni* and *C. coli* isolates to the various antibiotics with no significant differences in the resistance levels with the exception of nalidixic acid and norfloxacin. However, *C. coli* isolates were highly susceptible (0% of resistance) to norfloxacin and gentamicin. There are varied literature reports of resistance patterns of *C. jejuni* and *C. coli* strains; while some authors established higher resistance among *C. jejuni* (Tambur et al., 2009), others found higher resistance among *C. coli* (Jonker and Picard, 2010) and in some studies no difference in resistance were observed among the two species (Uzunovic et al., 2009; Ewnetu and Mihret, 2010). Moreover, no specific reasons have been cited for the differences in resistance among the two species (Luangtongkum et al., 2009).

Multidrug resistance of 100% was established in both faecal and carcass isolates which agrees with work in Malaysia by Tang et al. (2009) who recorded 100% MDR in poultry isolates. MDR rates of 35, 75 and 97% in poultry have, respectively been reported in Malaysia, Nigeria and Thailand (Sukhapesna et al., 2005; Akwuobu et al., 2010; Mansouri-najand et al., 2012). The observed high resistance rates against most of the assayed drugs may be explained by the reality of numerous disease outbreaks which frequently threaten the Ghanaian poultry industry (Aning, 1995) resulting in widespread use and abuse of antibiotics for prophylaxis and treatment of diseases and as growth promoters. The non-observance of withdrawal periods of antibiotics by Ghanaian poultry farmers which leaves residues in the poultry products with public health implications has also been reported by Turkson (2002). Chicken meat is a primary source of human campylobacteriosis, therefore the presence of antimicrobial-resistant *Campylobacter* in raw chicken meat constitutes a risk for consumers considering the high drug resistance found among isolates in this study.

Conclusion

Campylobacter species were present in the faecal and carcass samples of poultry birds (broilers and layers) at the Kejetia poultry slaughter in Kumasi. Contamination of carcasses by multidrug resistant *Campylobacter* strains poses risk to handlers and consumers. The abuse of antibiotics in poultry cannot be ignored deliberating the

high resistance levels documented against most of the commonly used antibiotics. Therefore constant education, surveillance and monitoring of antibiotic usage in poultry have become necessary.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Co-existence of multiple β-lactamase traits among clinical isolates of *Escherichia coli* from rural part of Maharashtra, India

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Acquisition of multidrug resistance and the spread of MDR among pathogenic microorganisms is a growing concern to world. Extended-spectrum β-lactamases (ESBLs) strains of the Enterobacteriaceae; ESBL trait expressing *Escherichia coli* poses major threat and ESBL *E. coli* infections are increasingly reported from hospital settings as well as community settings with rising mortality and morbidity rates. The objective of this study was to study distribution of various types of β-lactamases expressed within the clinical isolates of *E. coli*. In this work, clinical specimens: urine, stool, sputum, pus were collected over a period of three months from patients from rural area of Maharashtra State, India. Out of number of isolates, only thirty-six *E. coli* isolates were chosen for determining existence of ESBL, MBL, AmpC and Carbapenamase producing ability. Choice of isolates included resistance to at least of the three third generation cephalosprins used in this study. About 75% of *E. coli* strains were found to express ESBL trait, while remaining 25% expressed non-ESBL trait. About 28% isolates produced AmpC phenotype. Seven of 36 isolates (18%) indicated co-existence of ESBL and AmpC, while strain AKA43 was found to contain MBL and AmpC traits together. The prevalence of ESBL, MBL, AmpC and carbapenemase producing *E. coli* was studied. A few of these isolates were found to harbor more than one type of β-lactamases.

Key words: Extended spectrum ß-lactamases (ESBLs), ampCß-lactamases, metallo-ß-lactamases (MBLs), antibiotic resistance.

INTRODUCTION

The absence of new, effective anti-gram-negative antibiotics makes infection control the most important counter measure against multidrug-resistant gram-negative pathogens. Infection control can prevent

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additional infections and the spread of resistant pathogens and thereby reduce the need to use antibiotics (Anderson, et al., 2007; Arakawa et al., 2000; Bhavnani et al., 2006). Because susceptibility tests may be unreliable, special tests are required to detect the resistance mechanisms involved. The mechanisms include extended-spectrum-β-lactamases (ESBLs), AmpC β-lactamases and carbapenemases of molecular classes A and B (Anderson et al., 2007; Arakawa et al., 2000; Mohamed et al., 2006; Black et al., 2005; Ambler et al., 1991).

Extended-spectrum β-lactamases (ESBLs) strains of Enterobacteriaceae have emerged as significant pathogens (Rawat, 2010). Reports of infection or colonization with ESBL-producing Enterobacteriaceae strains have focused mainly on hospitalized patients or nursing home residents (Birgy et al., 2013; Paterson et al., 2005; Kenneth, 2010). Unlike most other anti-microbial-resistant pathogens that are associated with admission to hospitals, ESBL-producing *Escherichia coli* are predominantly community-onset pathogens (Cosgrove et al., 2002). Third world countries are considered as epicenter of this antimicrobial resistance primarily because the factors like overcrowding, poor nutrition and hygiene status and lack of infection control measures combined with antibiotic misuse/overuse has led to high endemic levels of resistant bacteria. Continuous and improper use of third generation cephalosporins has induced mutations in β-lactamases and has led to the emergence of ESBL producing Enterobacteriacae members (Fair, 2014; Arora, 2005).

Paterson (2005) stated that ESBL producing *E. coli* poses major threat and ESBL *E. coli* infections are increasingly reported from hospital settings as well as community settings with rising mortality and morbidity rates. Major risk factors for colonization or infection with ESBL producing organisms are long term antibiotic exposure, prolonged intensive care unit (ICU) admission, nursing home residency, severe illness, residence in surroundings with significant use of ceftazidime or other third generation cephalosporins (Ahammad, 2014). Besides these classical risk factors, the trait has been carried across the world by international travelers, food and animal trade form high to low prevalence region. Impact of international travelling is great; the best example is rapid spread of New Delhi Metallo β-lactmases (NDM-1) from India to other western countries (Kenneth, 2010). The Carbapenemases are diverse enzymes that vary in the ability to hydrolyze carbapenems and other β-lactams. Detection is a crucial issue because (i) they are often associated with extensive, sometimes total, antibiotic resistance and (ii) more-resistant organisms such as strains of *Pseudomonas* and *Acinetobacter* species that have acquired a carbapenemase can be vectors responsible for carbapenemase transmission to members of the family Enterobacteriaceae in which the resistance mechanism is not recognized (Thomson, 2010).

In this work, clinical specimens: urine, stool, sputum, and pus were collected over a period of three months from patients from slow progressing area of Maharashtra State, India. The objective of this study was to isolate and classify MDR *E. coli* straining exhibiting resistance to third generation cephalosporins, study distribution of various types of β-lactamases expressed within these clinical isolates of *E. coli*. To dissect what proportion of isolate make use of single and more than one types of β-lactamases.

MATERIALS AND METHODS

Bacterial isolates

Outdoor patients from the major hospitals of Barshi, Maharastra, India were grouped based on nature of samples and the symptoms. The clinical samples like urine, pus, stool and sputum were collected from patients for the microbial and biochemical analysis (Table 1). From these samples, bacteria were isolated by standard microbiological procedures and stored at 4°C for further experiments. Bacterial isolates were confirmed to be *E. coli* by VITEK-2 analyzer as well as standard biochemical properties as per Bergey's manual of systematic bacteriology.

Antibiotic susceptibility test

The bacterial isolates were screened for susceptibility to third generation antibiotics along with first and second generation β-lactams. The sensitivity of isolates were detected by Kirby-Bauer disc diffusion method (Bauer, 1966) on Mueller Hinton agar plates containing Ampicillin (25 µg), Oxacillin (1 µg), Penicillin-G (10 U), Ceftazidine (30 µg), Cefepime (30 µg), Tetracycline (30 µg), Cloxacillin (30 µg), and Cefotaxime (30 µg) (Himedia, Mumbai). The experiment was done as per the guidelines provided by Clinical and Laboratory Standards Institute (CLSI). Briefly, overnight grown culture density was adjusted to 0.5 MacFarland constant and plated on Mueller Hinton agar plates with sterile cotton swab. Four discs for antibiotics were placed on seeded agar plates. Zone diameter breakpoints recorded and organisms were classified as sensitive, intermediate and resistant as per the CLSI guidelines provided with *E. coli* NCTC 11954, strain harboring plasmid borne TEM-1 non ESBL type β-lactamase.

Detection of ESBL producers

Isolates exhibiting resistance to CTX, CAZ or CPD alone or together were tested for sensitivity in combination with clavulanic acid (CA). When inhibitory zones of CTX-CA, CAZ-CA or CPD-CA were found to be larger than 5 mm to that of zone for corresponding antibiotic without CA then these isolates were referred to as ESBL producers (Garrec et al., 2011).

Detection of MBL producer

The metallo β-lactamase (MBL) production was detected by combined disc method (Picao et al., 2008) using Imipenem (10 µg)

Table 1. Drug resistant bacterial isolates from hospital patients.

S/N	Species Name	Specimen	Gender
1	*E.coli*AKA41	Urine	F
2	*E.coli*AKA42	Pus	F
3	*E.coli*AKA43	Other	F
4	*E.coli*AKA44	Urine	M
5	*E.coli*AKA45	Urine	M
6	*E.coli*AKA46	Sputum	M
7	*E.coli*AKA47	Pus	F
8	*E.coli*AKA48	Pus	F
9	*E.coli*AKA49	Pus	M
10	*E.coli*AKA50	Urine	F
11	*E.coli*AKA51	Urine	M
12	*E.coli*AKA52	Pus	M
13	*E.coli*AKA53	Pus	M
14	*E.coli*AKA54	Pus	M
15	*E.coli*AKA55	Pus	M
16	*E.coli*AKA56	Pus	F
17	*E.coli*AKA57	Urine	M
18	*E.coli*AKA58	Urine	F
19	*E.coli*AKA59	Urine	F
20	*E.coli*AKA60	Pus	M
21	*E.coli*AKA61	Pus	M
22	*E.coli*AKA62	Urine	M
23	*E.coli*AKA63	Urine	F
24	*E.coli*AKA64	Urine	M
25	*E.coli*AKA65	Urine	F
26	*E.coli*AKA66	Pus	F
27	*E.coli*AKA67	Stool	F
28	*E.coli*AKA68	Urine	F
29	*E.coli*AKA69	Pus	F
30	*E.coli*AKA70	Urine	F
31	*E.coli*AKA71	Urine	F
32	*E.coli*AKA72	Urine	F
33	*E.coli*AKA73	Urine	M
34	*E.coli*AKA74	Stool	F
35	*E.coli*AKA75	Urine	F
36	*E.coli*AKA76	Urine	F

and Imipenem-EDTA. Both the IMP and IMP+EDTA discs were placed 30 mm apart from each other on seeded agar plates, plates were then incubated at 37°C for 24 h. Zones of inhibition were observed after 24 h. An isolate showing increase of inhibitory zone by more than 5 mm for IMP+EDTA than that of IMP alone considered to be MBL positive isolate, producing metallo β-lactamase enzyme.

Detection of AmpC producer

The AmpC production was detected by disc potentiation test using phenylboronic acid (PBA) as an enzyme inhibitor (Coudron, 2005; Tsakris et al., 2009). Phenyl boronic acid was dispensed onto commercially available Cefoxitin disc–CX (30 µg). These discs were dried and used within 30 min. The two discs CX and CX+PBA were placed on seeded agar plates by 30 mm apart from each other. An increase in inhibitory zone of ≥5 mm for the disc with PBA than that of CX alone was considered as an AmpC producer as the inhibitor potentiated the inhibitory effect of the cephalosporins by inactivating the AmpC enzyme.

Modified Hodge test for NDM1

The carbapenemase production among the isolates was detected

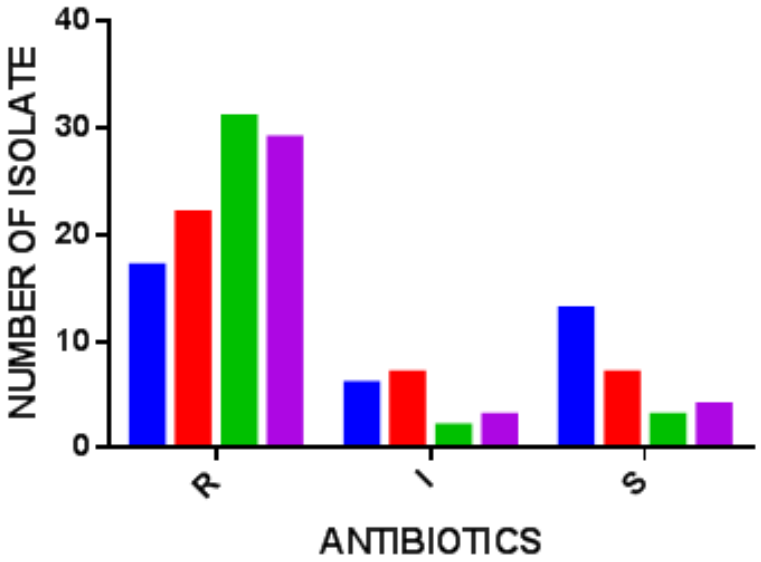

Figure 1. Antibiotyping for *E. coli isolates*. Antibiotic sensitivity estimated with disc diffusion assay. R denotes resistant phenotype, I denotes intermediate resistance while S denotes sensitive phenotype. The antibiotype expressed as histogram for various antibiotics; blue represents Tetracycline, red represent Cefepime, green represents Cefotaxime while purple indicates Ceftazidime.

by modified Hodge test as recommended by Clinical and Laboratory Standards Institute CLSI (Amjad et al., 2011). A lawn of 1:10 dilution of *E. coli* ATCC 25922 was spread on Muller Hinton agar plate seeded with *E. coli* strain ATCC 25922. Meropenem or Ertapenem (Dewees et al., 1970) (10 µg) susceptibility disc was placed in the centre of the ATCC 25922 seeded agar plate. In a straight line, test organism was spread from the edge of the disc to the edge of the plate. Plates were incubated at 37°C for 24 h. After incubation, the plates were examined for a clover-leaf type indentation at the intersection of the test organism and the *E. coli* ATCC 25922, within the zone of inhibition of the carbapenem susceptibility disc. The enhanced growth of *E. coli* ATCC 25922 toward the Ertapenem or Meropenem disc due to enzyme production by test organism was interpreted as a positive result for carbapenemase production.

Plasmid curing

Plasmid curing experiment was performed as described in Kharat and Mahadevan (1999). In brief, isolates exhibiting resistance to either Ceftazidime, Cefepime or Cefotaxime were subjected to cure the plasmid with low concentrations of ethidium bromide (EtBr). Overnight grown test organism 1:100 proportions to LB broth containing 10 µg/ml EtBr. Test organisms were incubated at 37°C for overnight. Grown cultures were serially diluted up to 10^{-6} with sterile physiological saline and 100 µl dilution was spread inoculated on LB agar plates. Plates were then incubated at 37°C for 24 h for the appearance of colonies. Well isolated colonies were picked and patched on LB agar medium plates supplemented with CTX (30 µg/ml) with a numbered grid line attached on the bottom of each plate. Plates were incubated at 37°C for 24 h. Plasmid was said to be cured from strain if a few of the isolated colonies could grow on LB agar plate but not on CTX agar plates, while parent strain could grow happily on LB agar and TCX agar plates.

Plasmid analysis

Plasmid DNA was isolated with standard alkali hydrolysis protocol. Plasmid were separated by agarose gel electrophoresis along with molecular weight marker and visualized under UV transilluminator. Purified plasmid DNA was used as donor to transform *E. coli* DH5α (NEB) with $CaCl_2$ method. Transformation mixture was plated on LB agar supplemented with 30 µg/ml CTX. The CTX^R transfromants were then tested for resistance to unselected antibiotics for which parent strain exhibited resistant phenotype (Upadhyay et al., 2015).

RESULTS

Susceptibility of *E. coli* isolates

Antibiotic susceptibility pattern for different β-lactams antibiotic was performed on *E. coli* isolates obtained from different clinical samples collected during May 2013- to July 2013. The group of antibiotics included were Penicillin, Ampicillin, Oxacillin, and Coxacillin, partially broad spectrum and third generation β-lactam members, namely, Ceftazidime, Cefotaxime and fourth generation Cefepime along with a non β-lactam protein synthesis inhibitor Tetracycline. The presence of ESBL, MBL, AmpC and carbapenemase activity by phenotypic confirmatory test was analyzed.

Majority of the isolates were obtained from female patients with urinary tract infection (urine) followed by sepsis (pus) sample (Table 1). Results shown in Figure 1

depict antibiotic susceptibility profile for all 36 *E. coli* isolates obtained from urine, pus, and stool. It is evident from Figure 1 that resistance to third generation cephalosporins was differential and found to be high ranging from 60 to 81% among the clinical isolates. Resistance to broad spectrum antibiotic Tetracyclin was comparatively low accounting for 47%.

ESBL producers

Out of these ESBL producers, 7 isolates were found to exhibit resistance to TET, accounting for tetracycling resistant strains to 16 out of 36 (Figure 1). The strain AKA46 and AKA49 were found to exhibit resistance to TET, CPM, CAZ, and CTX, whereas AKA50 and AKA54 were found to exhibit resistance to TET and CAZ; and TET and CTX, respectively. All nine isolates not exhibiting ESBL phenotype were also found to exhibit TET resistance phenotype (Figure 1). Resistance to selected antibiotics was differential, meaning β-lactamase producers were either resistant to one of the three cephalosporins and yet exhibited sensitivity to remaining two cephalosporins (Figure 1). Results represented in Figure 2 show that out of 36 *E. coli* isolates 75% could express ESBL trait while 25% were non-ESBL producers.

MBL and AmpC producers

Only AKA43 strain indicated existence of both MBL and AmpC (Figure 2B). In contrast, 10 isolates (28%) exhibited AmpC production ability (Figure 2A). Coexistence of ESBL and AmpC trait was observed in 7 isolates (Figure 2A). Coexistance of ESBL and AmpC trait in four of the seven strains is as shown in Figure 2C-1, strain AKA46, Figure 2C-2 for strain AKA51, Figure 2C-3 for strain AKA54 and Figure 2C-4 for strain AKA61. Interestingly, differential resistance to antibiotics was experienced among AmpC producers. Five of the AmpC producers were resistant to TET, seven were resistant to CPM, eight each were resistant to CTX and CAZ.

Carbapenem

Carbapenems such as imipenem and meropenem are powerful antibiotics that are not inactivated by ESBLs and AmpC lactamases and therefore are regarded as the last line of treatment for infections caused by ESBL producers. Recently, the rapid emergence and dissemination of carbapenem resistance in Enterobacteriaceae was reported due to production of carbapenemase enzyme. Presence of carbapenemase producers among the isolates was checked by Modified Hodge Test, none of the 36 isolates could exhibit carbapenamase production ability.

Plasmid curing

The EtBr is a mutagenic agent primarily causing frame shift by intercalating into the template DNA. The concentration required to impart mutation on genomic DNA is usually higher than it is required for low molecular weight extrchromosomal DNA. At lower concentrations, EtBr is known to interfere plasmid replication leading to plasmid loss in the next generations. We sought to test whether or not CTX resistance exhibited in clinical isolate is encoded on plasmid. The bacterial growth curve was performed at various concentrations to estimate lowest EtBr to be used in plasmid curing experiment. Bacterial growth was perturbed at 100 µg/ml EtBr, but unaffected at lower concentration, thus, we decided to use EtBr at 1/10 concentration (10 µg/ml) in plasmid curing experiment. Bacteria were grown and plasmid curing experiment was carried out as described in materials and methods. Plasmid curing was initially tested with replica plating for antibiotic markers; ceftazidime, cefepime and cefotaxime. All of the 36 clinical strains were able to gain sensitivity to cephalosporin antibiotics upon plasmid curing. These observations suggested that resistance exhibited in clinical isolates of *E. coli* is likely due to extrachromosomal genome.

Plasmid preparation

Existence of plasmid in clinical isolates was further substantiated as the demonstration of plasmid DNA. About eleven *E. coli* isolates were selected as sample for plasmid studies. Isolation of plasmid DNA with alkali lysis method was conducted on these 11 isolates. An aliquot of plasmid DNA was subjected for agarose gel electrophoresis. Results shown in Figure 3 indicate that all of the eleven plasmid preparations were successful substantiating observations of plasmid curing, which serves as second evidence. These observations are in agreement that cephalosporin resistance in *E. coli* isolates is likely due to expression of enzyme from the plasmid.

Transformation of antibiotic resistance

The DH5α cephalosporin naive competent cells were used as recipient and the plasmid DNA isolated from the earlier experiment served as the donor. Transformation mixture was plated onto LB agar supplemented with cefotaxime 30 µg/ml and incubated at 37°C for 24 h of the appearance of transformant colony. Experiments performed with controls indicated that plasmid DNA

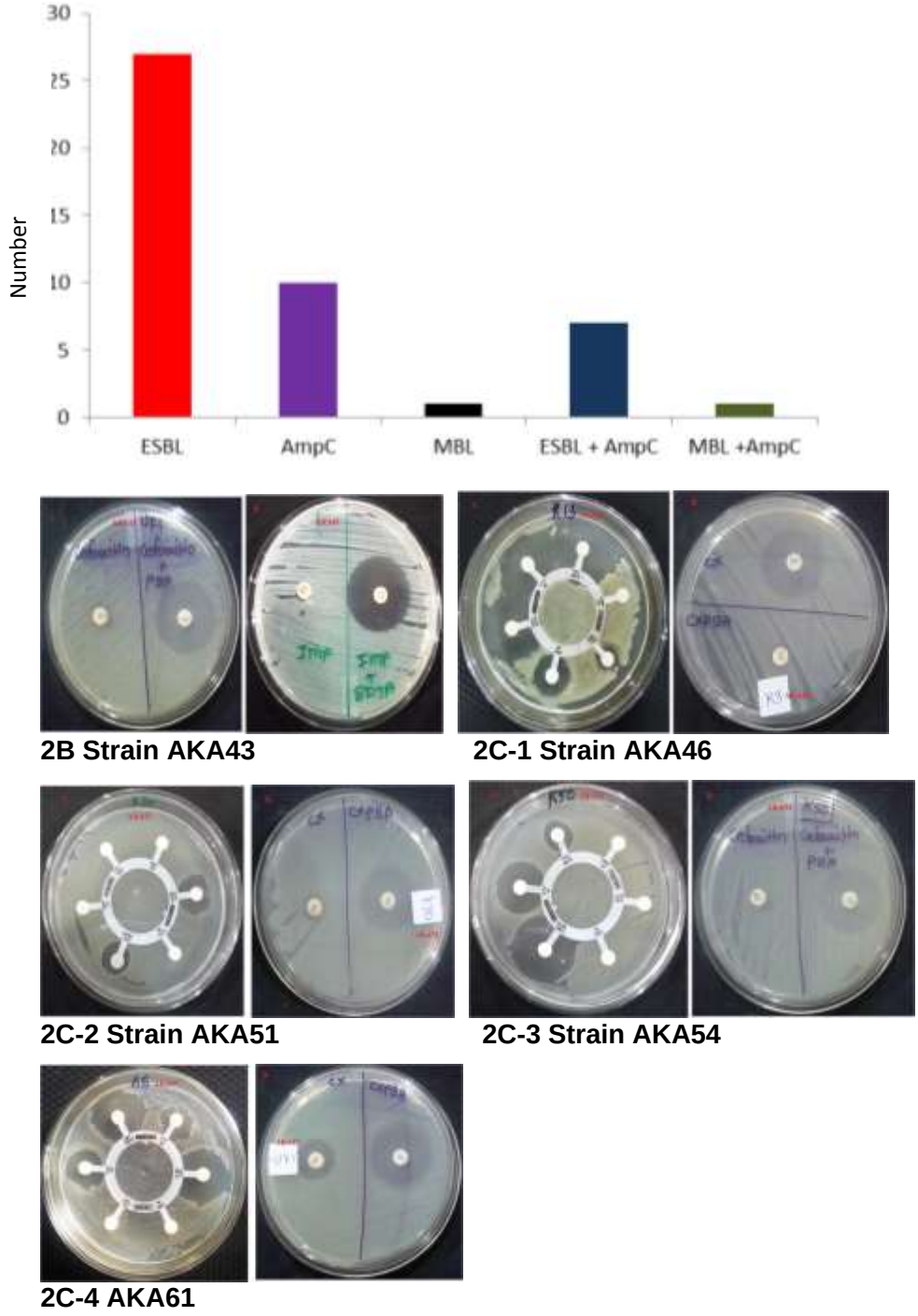

Figure 2. (A) Phenotypic classification of β-lactamase trait. Prescribed tests for detection of expression of ESBL, MBL, AmpC and Carbapenamase tests were conducted. In depicted histograms, red denotes ESBL, purple denotes AmpC, black denotes MBL, blue denotes dual ESBL and AmpC while green denotes MBL and AmpC expressing β-lactamase strains. (B) Phenotypic co-expression of MBL and AmpC trait in strain AKA43. Expression of the MBL was indicated by noticing enhanced zone of inhibition with Imp + EDTA whereas when enhanced with cefoxitin + phenylboronic acid it was treated as AmpC. (C) Phenotypic co-expression of ESBL and AmpC trait in 1. Strain AKA46, 2. Strain AKA51, 3. Strain AKA54 and 4. Strain AKA61. Expression of AmpC was indicated by noticing increased zone of inhibition for the disc with cefoxitin and phenyl boronic acid whereas when increased with cefotaxim + clavulanic acid it was treated as ESBL trait.

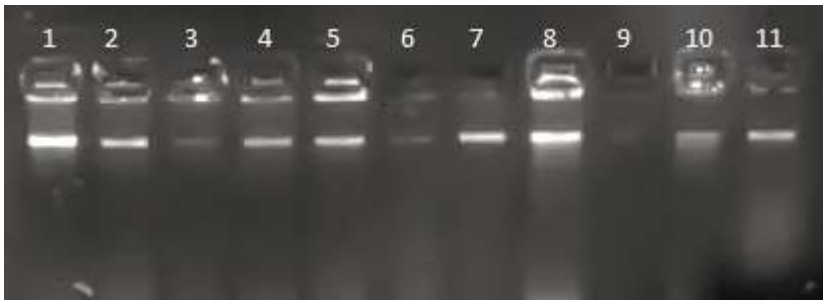

Figure 3. Plasmid analysis from a few □-lactamase producing *E. coli*. Plasmids isolated from the bacterial isolates were subjected for agarose gel electrophoresis. In the picture lane 1-AKA75, lane 2-AKA41, lane 3-AKA63,lane 4-AKA73, lane 5-AKA65, lane 6-AKA46, lane 7-AKA43, lane 8-AKA74, lane 9-AKA67, lane 10-AKA54, lane 11-AKA51, lane 1-AKA75, lane 2-AKA41,lane 3-AKA63,lane 4-AKA73, lane 5-AKA65, lane 6-AKA46, lane 7-AKA43, lane 8-AKA74, lane 9-AKA67, lane 10-AKA54, lane 11-AKA51.

isolated from eleven *E. coli* cephalosporin resistant strains was able to transfer CTX resistance. This experiment was again in agreement with results described in plasmid curing and yet provided another evidence to substantiate the proposition of cephalosporin resistance exhibited among 36 clinical *E. coli* isolates which is a plasmid borne trait.

DISCUSSION

ESBL detection is not routinely carried out in many microbiology laboratories of hospitals in developing countries. In our study, the prevalence of various β lactamases in the *E. coli* isolates was alarmingly high. The ESBL production was 75% found to be maximum as compared to the other β lactamases. Similar findings are in agreement with earlier reports (Bandekar et al., 2011) and 39.8% in burn ward patients (Ibrahim, 2013).

In this study, high resistance rates among ESBL-producing *E. coli* to first line antimicrobial therapy such as trimethoprim-sulfamethoxazole, tetracycline, nalidixic acid ciprofloxacin and ofloxacin were observed (data not shown). Similar findings have been reported in developing countries (Bandekar et al., 2011; Al-Muharrmi et al., 2008; Hussain, 2011; Idowu, 2011; Kader, 2004) as well as in developed countries (Moyo, 2010; Winokur, 2001). Significantly high rates of resistance to such commonly used oral antimicrobials have been previously described making these agents clinically ineffective for empirical treatment of infection caused by ESBL-producing strains (Kader, 2004; Moyo, 2010; Winokur, 2001; Ventola, 2015). Earlier studies reported that ESBL-producing isolates exhibited significantly higher resistant rates to non-β-lactamase antimicrobials agents including fluoroquinolones, aminoglycosides, tetracyclines and trimethoprim-sulfamethoxazole, compared to non-ESBL producing isolates. The possible explanation for this observation may be the fact that ESBLs are encoded on plasmids and can be mobile and therefore, easily transmissible as resistance gene elements for other antimicrobials from one organism to another (Bradford, 2001; Kader, 2004; Al-Agamy et al., 2006).

In this study, the AmpC production was seen in 27% isolates, which is in agreement with that of earlier AmpC producer reports. It was 17.3% in Kolkata (2005) and 22.9% in the study by Bandekar et al. (2011) from burn ward patients. In parallel studies, Bhattacharjee et al. (2008) reported that of total *Pseudomonas aeruginosa* isolates of 22% carrying AmpC trait. The only β-lactams which were active against the AmpC and the ESBL coproducers were the carbapenems. However, recently, the resistance to the carbapenems has been increasing, which is mostly due to the production of the metallo β-lactamases. In the index study, the MBL producers were 10.98%. In our studies, only strain AKA43 could express both MBL and AmpC traits.

Multidrug resistance producing Enterobacteriaceae strains in rural water reservoir in Guantao China (Zhang et al., 2015). About 42% ESBL-E (Enterobacteriaceae) carriers were detected from 18 villages of 3 counties located in Shandong, China (Sun et al., 2014; Chandel et al., 2011), while studying neonatal sepsis from rural and urban setting in India they identified the presence of ESBL producing strains in community of infants with no prior history of hospitalization or antibiotic use. Similar to our methodology and observations, co-existence of ESBL and MBL was confirmed with phenotypic and VITEK 2 advanced expert characterization by (Kotwal et al., 2016). In their studies of total *Pseudomonas aeruginosa* strains, only 2% strains could co-express ESBL and MBL. In the present study, the AKA43 strain could exhibit expression of two traits, but MBL and AmpC.

In our study, the occurrence of ESBL-producing *E. coli*

among urine specimens is of great concern, since *E. coli* is one of the main causative agents of urinary tract infections and consequently due to the use of antimicrobial agents, ESBL producing *E. coli* is more likely to spread. This was also true in the present study, because prevalence of ESBL producing *E. coli* isolates varied among the participating hospitals from 45.1 to 75%. Although, earlier studies from different countries have reported that ESBL producing Enterobacteriaceae, *P. aeruginosa*, *Klebsiella pneumoniae* and *E. coli* may be found in rural area, this is a first report from rural part of Maharashtra, Western India. This article collectively describes that *E. coli* isolates not only exhibit resistance to third generation cephalosporins, but also co-expression of at least two β-lactamases. These observations express dire need of cautious antibiotic prescription to be avoided when not indicated on the basis of clinical microbiology investigation.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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Full Length Research Paper

Post-treatment of cachaca (Brazilian sugarcane spirit) with charcoal made from cane bagasse

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The objective of this study was to ferment sugarcane juice using two types of yeast and evaluate the quality of the distillate after beverage filtration in a column filled with ground charcoal derived from cane bagasse. The experimental design was randomized block (split plots, 2 x 2) with three replicates. The primary treatment involved two types of yeast: CA-11 and baker's yeast. Secondary treatment involved filtration or not of the beverage in the charcoal column. The following procedures were adopted: percolation of all samples to a single column and passage of the distillate from each repetition (blocks) using three similar columns. Selected yeast had similar cachaça production as compared to the baker's yeast. However, higher acidity occurred in the distillate using the selected yeast, which contributed to the increased transfer of copper to the beverage. In the charcoal filtration that used one column per block, the copper was removed from the beverage, and the acidity was decreased, whereas the alcohol content, fixed acidity, pH, and turbidity of the cachaça did not change.

Key words: Spirits, yeast strains, fermentation, distillation, filtration columns.

INTRODUCTION

Cachaça (Brazilian sugar cane spirit) is a distillate produced in Brazil and appreciated throughout the world (Borges et al., 2014). This distillate is obtained by the fermentation of sugarcane juice and needs to have an alcohol content between 38 and 48% $(v/v)^{-1}$ (Brazil, 2005). It is the third most consumed distillate in the world after vodka and soju (Kunigk et al., 2011). Despite the high yield and the import from Brazil to more than 60 countries, the export is still insignificant, corresponding to a little more than 1% of the total production. One of the reasons for the limited export includes the variation in quality and copper content higher than 2 mg L^{-1} (Lima et al., 2009).

After the alcoholic fermentation of sugarcane juice, distillation is performed mainly in copper stills (Alves et al., 2011) to reduce the acidity and the content of aldehydes and sulfur compounds in beverages (Pereira et al., 2012). However, during distillation, cachaça may

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be contaminated with metals ions removed from the distiller.

Moreover, some compounds produced by yeasts during fermentation, particularly organic acids, may promote the transfer of copper from the stills to the beverage (Boza and Horii, 2000). During the fermentation process, several yeast types can be used, including baker's yeast (pressed), which is readily available and fast-growing, and selected yeast, which is adapted to the production of cachaça. Although, these strains belong to the same species (*Saccharomyces cerevisiae*), they produce wines with different ethanol, acidity and secondary compounds concentration (Basso et al., 2011). One of the yeast strains selected for cachaça production is CA-11.

Alcarde et al. (2012) found that strain CA-11 provided the most appropriate chemical composition to the distillate compared with the other strains studied (Y-904, BG-1, PE-2, SA-1 and CAT-1). Action of CA-11 is different from other strains during fermentation. Montijo et al. (2014) did not correct the pH of the must and inoculated CA-11 directly into the must because, according to the authors, the acids produced during fermentation lowered the pH of the must. The lowering of the pH during fermentation is necessary to promote the increased use of sugars by the yeasts rather than by contaminating bacteria (Basso et al., 2011). However, during distillation, the improper sanitation of the distiller and the high concentration of acid in wine favors the transfer of the verdigris [$CuCO_3.Cu(OH_2)$] to the beverage (Boza and Horii, 2000).

The cachaça samples obtained in different Brazilian states have a high percentage of copper. The copper content of the beverages analyzed in southern Minas Gerais was higher than that recommended in 16.67% of the samples, and the content in one evaluated sample was as high as 9 mg L^{-1} (Zacaroni et al., 2011). Marinho et al. (2009) observed that the copper content in the cachaça produced in Goiás was as high as 8.80 mg L^{-1}. The average levels of copper in the beverages produced in Rio Grande do Sul ranged between 2.95 and 10.22 mg L^{-1}, and depending on the region, up to 60% of the samples were contaminated (Garbin et al., 2005) by exceeding the copper level limit of 5 mg L^{-1} established by Brazilian legislation (Brazil, 2005).

Several methods can be used to remove organic and ionic contaminants from cachaça. For copper removal, the main techniques include adsorption on activated carbon and ion exchange resins (Lima et al., 2006; Kunigk et al., 2011), adsorption in activated carbon/iron oxide composites (Lima et al., 2009), and double distillation (Alcarde et al., 2012), among others. However, the efficiency of the removal of metals and other undesirable components could be improved by converting the bagasse into charcoal, and use this for cachaça filtration.

Charcoal has a high adsorption capacity. Several studies have evaluated the efficacy of charcoal as an adsorbent, including its use for the retention of organic compounds such as trihalomethanes, nitrates, chloride ions, sulfide ions, metals (Pb, Cu, and Fe), and microorganisms such as fecal coliforms and total coliforms (Ta-Chung et al., 2008). Zhao et al. (2008) evaluated the adsorption of 2,4-dichlorophenol to charcoal (Xu and Shi, 2002) and the elimination of nitrates (Mizuta et al., 2004) and four types of phthalate esters in water.

Charcoal from sugar maple wood (*Acer saccharum*) has been used to remove fusel oil from whiskey by filtration using the charcoal mellowing process (Labbe et al., 2006). The charcoal produced from sugarcane bagasse also has adsorption capacity. Teixeira et al. (2016) used bagasse charcoal for filtration and the removal of solids from vinasse, which was subsequently used as dilution water for the preparation of the must of molasses, a waste product of sugar production.

However, the use of sugarcane bagasse charcoal for the filtration of alcoholic beverages has been little explored. The possibility of using charcoal for the removal of toxic components present in the beverage is interesting and is an alternative use of bagasse.

In this context, this study aimed to compare the quality of cachaça obtained after distillation of must fermented using baker's and selected yeasts and to investigate the final quality of the beverage after filtration using a column filled with ground charcoal produced from ground sugarcane bagasse.

MATERIALS AND METHODS

Experimental design

The experimental design was randomized block (split-plot) with three replicates. The primary treatment involved the use of two types of yeast for the production of cachaça: selected yeast and baker's yeast. Secondary treatment was performed after fermentation and distillation, with the filtration or not of the cachaça in a column containing ground charcoal produced from sugarcane bagasse.

The experiment was conducted in blocks to distinguish the different days of fermentation (March 21, 22, and 29, 2016). The sugarcane juice was extracted from sugarcane variety IAC95-5000, with 11 months, and obtained from the first cut.

Fermentation: Preparation, treatment and analysis

Soluble solids content (°Brix), pH, acidity and total reducing sugars (TRS) were determined in the must used in fermentation, as established in the *Centro de Tecnologia Canavieiro* CTC (2011). For fermentations, the following yeast strains were used: baker's *Saccharomyces cerevisiae* (Levasaf®, Argentina) and selected yeast CA-11 (LNF®, Brazil).

The original total cell count and cell viability were determined for each type of yeast with Ringer's solution in a Neubauer chamber, as detailed by Lee et al. (1981). Subsequently, based on the cell viability, the amount of yeast to be added in order to reach a concentration of 10^7 CFU mL^{-1} of yeast cells during fermentation

Figure 1. Fifteen-liter still used for the distillation of the fermented must.

was calculated.

Initially, yeast was diluted in must (2500 mL) that was used in the fermentation process. The feeds with 2500 mL of must, with temperature of 32°C, were timed every 15 min until the Fed Batch fermentation vat reached a volume of 10,000 mL. Fifteen minutes after the last feed, samples were collected for another evaluation of initial cell viability. Fermentation was completed after 24 h (Oliveira et al., 2008), when soluble solids (°Brix) was less than 1 (one). Then, wine samples were collected for the assessment of final cell viability and execution of the analyses.

Analysis of wine samples

Sulfuric acidity (g H_2SO_4 L^{-1}), alcohol content (% vv^{-1}), glycerol (10^{-3}%, wv^{-1}), total residual reducing sugars (TRRS - %), and pH were analyzed according to the CTC (2011). The amount of alcohol produced in wine was calculated by taking into account the amount of wine produced multiplied by the alcohol content. The fermentation efficiency (%) was calculated by dividing the amount of alcohol produced by the theoretical amount of alcohol produced, as detailed by Fernandes (2006).

Distillation

The wine was distilled in copper stills (D&R Alambiques®, Belo Horizonte, Brazil) (Figure 1), and three fractions were obtained: the first fraction (10%) with mainly head compounds (aldehydes and esters), a major fraction (80%) that is considered the heart (cachaça), and the last fraction (10%), that is, the tails compounds (phurphural and others not desirable components) (Bruno, 2012). The last fraction was not separated and remained in the vinasse because the distiller was switched off after the separation of the heart fraction. Distillation was initially conducted in the wine produced with the selected yeast, followed by the wine produced with the baker's yeast. The order of the type of yeast was changed in the other replicates. Before each distillation, the equipment was cleaned with water only. The fractions removed from the equipment were maintained at room temperature in glass bottles covered with aluminum foil. The amount of material to be separated in the heart fraction was calculated to obtain a beverage with an ethanol content of approximately 40% at the end of the distillation.

Figure 2. Charcoal column used for filtration of the cachaça obtained from fermented must produced with the selected and compressed yeast.

Charcoal production and treatment of cachaça

Charcoal was produced using sugarcane bagasse. The bagasse was carbonized at 250°C for 1 h, and then at 400°C for 1 h in an adapted (Machado et al., 2014) muffle (Tecnal®, Piracicaba, Brazil). The process was repeated until a sufficient amount of charcoal was obtained for making the filtration columns. The yield of charcoal production was approximately 30%.

The glass column used was 35 cm in height and 5 cm in diameter, with 500 mL of useful volume with markings (Figure 2). The charcoal was ground and sieved through a 35-mesh sieve (0.5 mm) and placed in the column. Cotton was added (35 mL – this grading was used as a reference for the mounting of other columns) to keep the charcoal particles in place inside the column during filtration. A filter layer was made with the ground and sieved charcoal (95 mL), and cotton (25 mL) was placed above this layer. A layer of crushed charcoal (55 mL), which corresponded to the prefilter layer, was stacked above this cotton layer. Another layer of cotton (30 mL) was placed above the prefilter layer to prevent the spreading of the crushed charcoal layer during the addition of the beverage. The total final volume of the filter layer was 240 mL and this procedure was repeated for every column filtration build.

The column was packed using commercial cachaça (51®, Pirassununga, Sao Paulo, Brazil) to settle the filtering layers of charcoal and cotton. For beverage filtration, 260 mL of each replicate was passed through the entire column and then discarded. Another fraction of 260 mL was filtered; this fraction was considered the analyte. This procedure was followed for each sample. Filtration was performed in the dark at room temperature (approximately 25°C). The residence time for percolation of 260 mL of cachaça in the column was approximately 15 min.

An aliquot of the heart fraction was filtered with charcoal two months after storage in the bottle. Two ways of filtration were conducted as follows: a) use of a single charcoal column for passage of all three replicates for both yeasts and b) use of one

Table 1. Average and standard error of pH, soluble solids (°Brix), acidity (g H_2SO_4 L^{-1}), and TRS (%) from three replicates of the cane juice used as must for the production of cachaça.

Analysis of the cane juice	Mean and standard error	Recommended*
pH	5.25±0.07	4.5 to 5.5
°Brix (%)	16.93±0.96	≥ 18
Acidity (grams of H_2SO_4 L^{-1})	2.31±0.27	≤ 0.8
TRS (%)	14.33±0.79	≈ 18

*Amorim et al. (1996).

filtration column per block (replicate). The order of passage of the type of yeast was switched for each block. In the first block, the selected yeast was used first, followed by the baker's yeast. Then, another column was made and changed: cachaça from bakers filtered first and yeast selected after. And the ultimate column was prepared for third repetition, and passed spirits from selects followed by baker's yeast.

The variables pH, alcohol content, turbidity (MS Tecnopon, TB1000, Brazil), total acidity, fixed acidity, and acetic acid were measured in the distillate (treated or not) according to the protocols of the Adolfo Lutz Institute (2008). The copper content was determined only in samples that were passed through the different columns per block. The copper content was determined using atomic absorption spectroscopy in an AAnalyst800 spectrometer (Perkin Elmer®, USA) with an air-acetylene flame and the standard addition method. The following analytical conditions were used: wavelength of 324.8 nm, air flow rate of 17.0 L min^{-1}, acetylene flow rate of 2.2 L min^{-1}, and burner slot width of 0.7 nm.

The copper content of one of the samples, which had concentrations below the detection limit of the flame, was determined using atomic absorption spectroscopy with a graphite furnace and the standard addition method. For this purpose, an AAnalyst800 spectrometer (Perkin Elmer, USA) and the following analytical conditions were used: wavelength of 324.8 nm, pyrolysis temperature of 1200°C, atomization temperature of 2200°C, and chemical modifier consisting of 5 µg of Pd and 5 µg of Mg. The use of a chemical modifier allows the use of high temperatures to remove the matrix, prevents the loss of the analytes, decreases the volatility of the analytes, and permits the separation of the analyte from the matrix.

Statistical analysis

The quality of the must, fermentation, and wine were compared by evaluating the differences between the means with standard errors. The results obtained from only filtration or in three blocks columns were subjected to analysis of variance using the F-test and Tukey test ($p \leq 0.05$), as detailed by Barbosa and Maldonado Jr. (2015).

RESULTS AND DISCUSSION

Fermentation

The analysis of the must (Table 1) indicated that the sugarcane variety used was not yet at peak maturation because the soluble solids and TRS values were lower than expected for the production of cachaça and the acidity was higher than 0.8 g H_2SO_4 L^{-1} (Amorim et al., 1996). This result was expected because sugarcane was harvested purposely before the beginning of the harvest season (from May to November for sugarcane in Brazil) (Oliveira et al., 2008). In March, performing the fermentation and producing high-quality distillates would be more difficult. However, this condition was ideal to test the method of removal of impurities from the beverage using charcoal. The sugar content and pH of the cane juice were not adjusted to simulate what, in practice, occurs during the production of cachaça using a nonstandard must. However, the must was the same for both types of yeast in each block (replicate).

Table 2. Average and standard error of three replicates for the initial and final cell viability (%) in the fermented must using selected and pressed yeast for production of cachaça.

Yeast type	Cell viability	
	Initial (%)	Final (%)
Selected	87.84±1.89	68.04±7.17
Baker's	79.79±5.87	38.30±14.21

The high acidity of the must significantly decreased the yeast cell viability during the fermentation process (Table 2). The original viability of the baker's yeast was lower than that of the selected yeast. The cell viability of the baker's yeast was below the recommended value of 85% (Amorim et al., 1996). Previous calculations were performed to avoid the shortage of yeast in fermentation because the original cell viability of the baker's yeast was low (77.61%), whereas the viability of the selected yeast was 81.65%. For this reason, no shortage of yeast occurred because of addition of sufficient cells to have 10^7 viability cells for both strains.

Viability remained low at the beginning of fermentation (Table 2). At the end of fermentation, the decrease in cell viability of both types of yeasts was higher which could prevent the reuse of the yeast in new fermentation cycles. In these cases, the addition of a new batch of yeast would be required. Furthermore, a higher precipitation of the selected yeast was observed after fermentation, which made the separation of the wine from the yeast easier. The same result was obtained by

Table 3. Average and standard error of three replicates regarding pH, alcohol content (% v v^{-1}), glycerol (10^{-3}% w v^{-1}), acidity (H_2SO_4 g L^{-1}), total residual reducing sugar TRRS (%), and fermentation efficiency (%) in the fermented must obtained with the selected and pressed yeast for cachaça production.

Yeast type	pH	Alcohol content	Glycerol	Acidity	TRRS	Fermentation efficiency
Selected	3.99±0.04	9.57±0.26	0.83±0.07	3.12±0.16	0.63±0.19	93.26±5.77
Baker's	3.99±0.04	9.41±0.34	0.89±0.05	3.19±0.28	0.62±0.33	91.44±3.14

Table 4. Average and standard error of three replicates regarding alcohol content (% v v^{-1}) in the first fraction, major fraction, and vinasse + last fraction, and volume (mL) of ethanol present in the stills (adjusted to 40% alcohol content) for the production of cachaça using two types of yeast.

Yeast type	Alcohol content			Total volume	Volume adjusted to 40%
	First fraction	Heart fraction	Vinasse + last fraction		
Selected	47.80±1.61	44.23±0.44	2.41±0.19	1416.67±83.33	1567.50±101.28
Baker's	45.49±4.81	46.17±2.44	1.66±0.60	1333.33±66.67	1542.75±120.16

Table 5. Analysis of variance for total acidity (g of CH_3COOH 100 mL^{-1} of sample), fixed acidity (g of CH_3COOH 100 mL^{-1} of sample), volatile acidity (mg of CH_3COOH 100 mL^{-1} of anhydrous ethanol), pH, alcohol content (% v v^{-1}), and turbidity (NTU) for cachaça produced using two yeast strains and filtered or not in a single charcoal column for all replicates.

Causes of variation	Total acidity	Fixed acidity	Volatile acidity	pH	Alcohol content	Turbidity
Blocks	1.09^{ns}	2.07^{ns}	0.05^{ns}	3.41^{ns}	3.92^{ns}	6.45^{ns}
Yeast (L)	46.35*	2.42^{ns}	18.86*	10.78^{ns}	2.89^{ns}	0.04^{ns}
Selected	35.90±3.44 ^{A}	4.81±1.09^{A}	69.90±6.44^{A}	5.15±0.08^{A}	44.49±0.51 ^{A}	1.33±0.24 ^{A}
Baker's	17.95±1.70^{B}	2.90±0.83 ^{A}	32.70±4.33 ^{B}	5.43±0.07^{A}	46.19±0.61 ^{A}	1.31±0.28 ^{A}
CV of the fraction	16.96	55.13	28.92	2.79	3.82	13.85
Charcoal (T)	1.14^{ns}	10.42*	7.99*	3.16^{ns}	0.59^{ns}	4.07^{ns}
With treatment	25.07±2.86^{A}	5.69±1.14 ^{A}	42.84±6.40 ^{B}	5.20±0.11^{A}	45.48±0.52 ^{A}	0.95±0.26 ^{A}
Without treatment	28.78±2.28^{A}	2.02±0.78 ^{B}	59.77±4.37 ^{A}	5.39±0.02^{A}	45.20±0.60 ^{A}	1.69±0.26 ^{A}
Interaction (L x T)	2.24^{ns}	1.67^{ns}	6.82^{ns}	5.04^{ns}	0.39^{ns}	0.18^{ns}
CV of the sub-fraction	22.39	51.08	20.22	3.50	1.43	48.49

**Significant at a probability of 1% using the F-test; ns: not significant; CV: coefficient of variation (%). The same letter in each column indicates the absence of a significant difference using Tukey test ($p \leq 0.05$).

Alcarde et al. (2012). Yeast flocculation is desired during the production of cachaça (Basso et al., 2008) because poor separation of the wine from the yeast may increase the amount of yeast present in the distiller, which in turn increases the concentration of fatty acids in the distillate (Serafim et al., 2011).

A slight decrease was observed in the glycerol content in the wine produced with the selected yeast as compared to the wine produced with the baker's yeast (Table 3). Furthermore, using standard error, pH, alcohol content, acidity, TRRS and fermentation efficiency was similar for both strains.

The alcohol content of the first (initial 10% that was separated from the beverage) and heart fraction was similar for both strains (Table 4). However, the last fraction + vinasse have more alcohol content for select yeast. This result lead us to affirm that more alcohol could be recovered and it was lost. Total volume of the produced distillate (heart fraction) was higher in select yeast; however, if the alcohol content was standardized to 40% (Table 4), the volume of the heart fraction was similar between strains. Baker's and selected yeast had close performance for cachaça production.

Filtration of all replicates using a single charcoal column

After the distillate filtration of all replicates using a single column, the volatile acidity was 2.13-fold greater for the selected yeast, although the acidity obtained using the two types of yeast was within the values recommended by Brazilian legislation (up to 150 mg of acetic acid per 100 mL of anhydrous alcohol) (Brazil, 2005) (Table 5).

The yeast strain has been shown to affect the chemical composition of the distillate (Alcarde et al., 2012).

Table 6. Analysis of variance for total acidity (g of CH_3COOH 100 mL^{-1} of sample), fixed acidity (g of CH_3COOH 100 mL^{-1} of sample), volatile acidity (mg of CH_3COOH 100 mL^{-1} of anhydrous ethanol), pH, alcohol content (% v v^{-1}), and turbidity (NTU) in cachaça produced with two types of yeast and filtered or not in a single charcoal column per replicate.

Variation causes	Total acidity	Fixed acidity	Volatile acidity	pH	Alcohol content	Turbidity	Copper content
Blocks	9.18^{ns}	11.59^{ns}	3.59^{ns}	0.04^{ns}	2.17^{ns}	4.88^{ns}	0.43^{ns}
Yeast (L)	259.89**	3.28^{ns}	95.50*	3.01^{ns}	1.64^{ns}	3.35^{ns}	7.81^{ns}
Selected	32.39±1.44 ^{A}	4.57±1.46 ^{A}	62.90±3.36 ^{A}	5.14±0.12 ^{A}	44.25±0.61 ^{A}	1.51±0.29 ^{A}	12.66±1.04 ^{A}
Baker's	15.03±1.57 ^{B}	3.31±1.21 ^{A}	25.44±2.33 ^{B}	5.54±0.19 ^{A}	45.82±0.84 ^{A}	1.91±0.48 ^{A}	7.11±1.13 ^{A}
CV of the fraction	7.87	30.57	15.02	7.37	4.75	22.16	34.82
Charcoal (T)	24.15**	4.35^{ns}	74.15**	2.03^{ns}	0.28^{ns}	0.01^{ns}	83.76**
With treatment	18.64±1.21 ^{B}	5.86±1.36 ^{A}	28.58±1.51 ^{B}	5.49±0.21 ^{A}	44.88±0.83 ^{A}	1.74±0.40 ^{A}	3.82±1.21 ^{B}
Without treatment	28.78±1.80 ^{A}	2.02±1.32 ^{A}	59.77±4.19 ^{A}	5.20±0.10 ^{A}	45.20±0.62 ^{A}	1.69±0.37 ^{A}	15.95±0.96 ^{A}
Interaction (L x T)	7.90*	0.20^{ns}	18.03*	0.37^{ns}	0.35^{ns}	0.23^{ns}	34.85**
CV of the subfraction	15.07	80.97	14.20	6.64	2.31	55.76	23.24

**Significant at a probability of 1% using the F-test; ns: not significant; CV: coefficient of variation (%). The same letter in each column indicates absence of significant difference using Tukey test ($p \leq 0.05$).

Depending on the acids produced during fermentation, the concentration of these acids can be higher in the first or last fraction. Accordingly, the concentration of acetic acid, succinic acid, glycolic acid, citramalic acid and lactic acid was much higher in the last fraction, whereas capric acid, lauric acid, myristic acid, and palmitic acid were more abundant in the initial fractions (Serafim et al., 2011). Alcarde et al. (2012) studied the yeast strain CA-11 in double distilled cachaça and found that volatile acidity in the first, heart and last fractions was 14.20, 23.67 and 124.51 mg 100 mL^{-1}, respectively, indicating that the volatile acid content was higher in the last fraction.

The balance of the types of acids, alcohol content of the wine, and other secondary compounds from fermentation are essential to define when they will be removed from the wine during the distillation process. The baker's yeast could produce a higher percentage of fatty acids than the selected yeast considering that the separation between the yeast fraction and the wine was more difficult, and therefore, more yeast was added to the distiller. Because of the higher boiling point of fatty acids, the removal of organic acids may have changed for the beverage obtained with the baker's yeast, resulting in lower concentrations of these compounds in the heart fraction.

The total acidity was similar between the beverages in a single filtration column using charcoal; however, it was significantly different for the fixed and volatile acidity (Table 5). The fixed acidity increased 2.82-fold after filtering with charcoal whereas the volatile acidity decreased by 40% in the filtered distillate. The increase in fixed acidity may be due to the presence of iron and aluminum in the charcoal because bagasse ashes have 6.87% of Fe_2O_3 and 7.48% of Al_2O_3 on average (Rodriguez-Diaz et al., 2015). Metal ions with high charge and small volume, such as Fe^{3+} and Al^{3+}, in aqueous medium, in the form of $[Fe(H_2O)_6]^{3+}$ ($pKa = 3.5 \times 10^{-3}$) and $[Al(H_2O)_6]^{3+}$ ($pKa = 1.4 \times 10^{-5}$), respectively, can act as Lewis acids and produce acidic solutions (Atkins and Jones, 2006).

Passage of the distillate using one column per block

Differences in fixed acidity were not significant between the treatments subjected or not to filtration with charcoal (Table 6). However, there was an interaction among the yeast type and charcoal filtration for total acidity (Figure 3A) and volatile acidity (Figure 3B).

The difference in total acidity after filtration with charcoal was more pronounced for the beverage from selected yeast (Figure 3). The acidity for the selected yeast decreased significantly (by 65.3%) after filtration, and the values obtained with the selected yeast were closer to those obtained with the baker's yeast. No significant difference in total acidity after filtration with charcoal was observed for the baker's yeast. The total acid content of the beverage produced with this type of yeast was low before and after filtration.

Volatile acidity is a parameter used in Brazil to assess the quality of cachaça decrease in beverages produced with each type of yeast after filtration (Figure 3B). This reduction reached 2.8 and 1.9-fold using the selected and baker's yeast, respectively, after filtration. Moreover, the volatile acidity value of the beverage produced with the selected yeast after filtration (39.61mg) was similar to that obtained using baker's yeast (33.35 mg of acetic acid) before filtration.

The copper content found in the unfiltered beverages was very high (Table 6). The cachaça samples with excess copper present levels between 8 and 10 mg L^{-1}

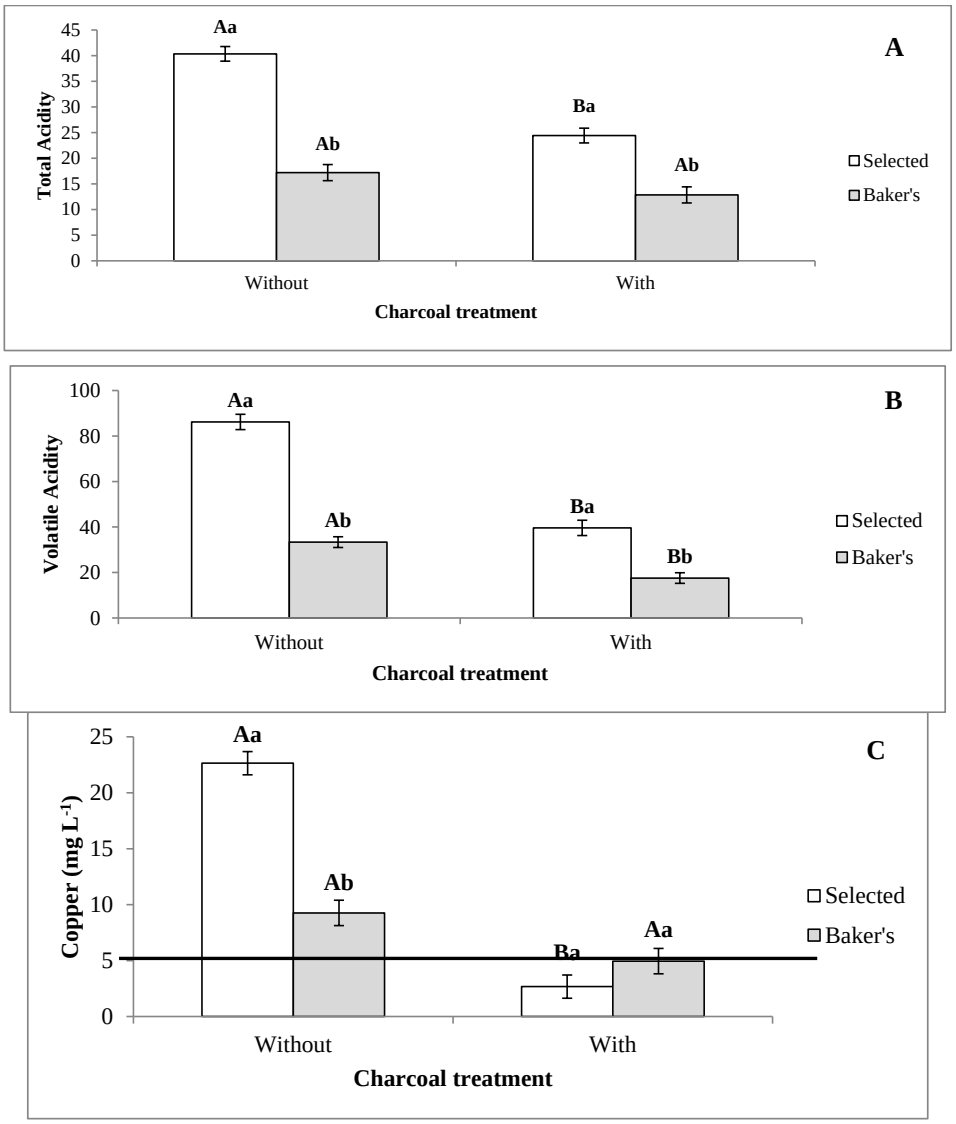

Figure 3. Filtration using a single charcoal column per replicate of cachaça produced. A. Total acidity (g CH_3COOH 100 mL^{-1} of sample); B. Volatilty acidity (mg of CH_3COOH 100 mL^{-1} anhydrous ethanol); and C. Copper content (mg L^{-1}). Bold black line indicates the maximum copper level allowed by Brazilian legislation (5 mg L^{-1}). Bars are the standard error. Capital letter compares filtration in each type of yeast and lower case letters compare strains in each kind of filtration. Same letters did not differ by Tukey test ($p \leq 0.05$).

(Lima et al., 2006) or more. Negri et al. (2015) found copper levels of up to 28 mg L^{-1} in the evaluated distillates. The copper content in samples prepared with must fermented with the selected yeast was up to 25.16 mg L^{-1} (Figure 4B). This result might be because the still used was made of copper and the low cleaning efficiency of the still, which increased the levels of copper above those permitted by Brazilian legislation. For the baker's yeast, the maximum level found was 10.68 mg L^{-1}. The acids help to drag the verdigris to the beverage (Boza and Horii, 2000). The increase of the metal in the beverage might have occurred because the selected yeast produced a higher amount of total acids in the distillate (Table 6).

After filtration, the copper concentration (15.95 mg L^{-1}) decreased 4.17-fold to 3.82 mg L^{-1} (Table 6). However, the effect of the interaction indicated the cachaça produced with the selected yeast, which contained higher copper levels due to discharge from the still, the decrease was even higher (8.45-fold) (Figure 3C). This effect occurred because of the order of passage of the beverage replicates inside the charcoal column (Figure 4). For the baker's yeast, copper level decreased from 8.885 to 0.248 mg L^{-1} in the filtered sample (Figure 4B).

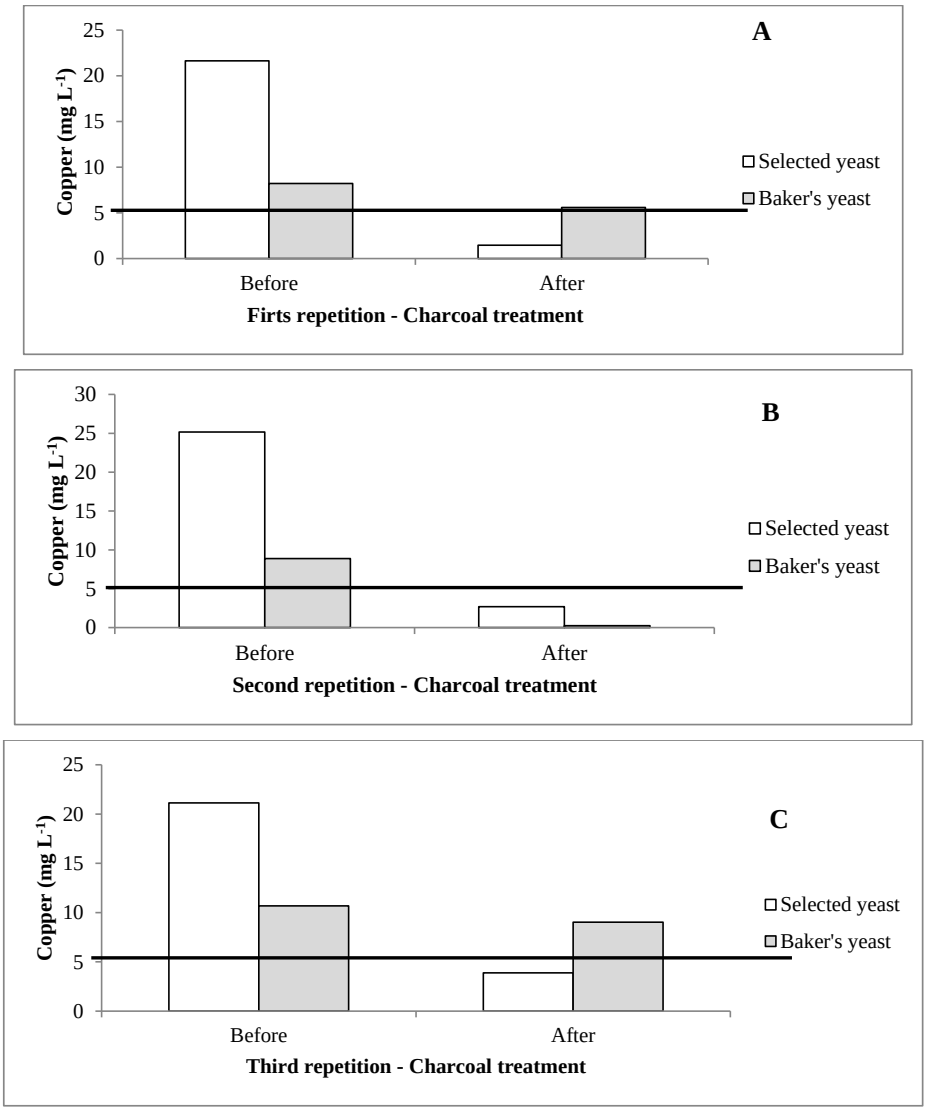

Figure 4. Filtration of cachaça using a single charcoal column per replicate. A. Filtration was performed in the beverage produced with the selected yeast and then in the beverage produced with the baker's yeast. B. In the second replicate, filtration was performed in the beverage produced with the baker's yeast, followed by selected yeast. C. In the third replicate, filtration was performed in the beverage produced with the selected yeast and then with baker's yeast. The bold black line indicates the maximum value allowed by the Brazilian legislation (5 mg L^{-1}).

The charcoal column removed high amounts of metal but presented problems of saturation (Figure 4). Therefore, the correct procedure would be mounting one column per sample to decrease the amount of copper in the cachaça to internationally acceptable levels.

No changes in the amount of ethanol in the beverages or in pH and turbidity were observed, as possibility of dragging of the charcoal. Bagasse would be discarded or used for burning or heating of the still during distillation. This residue is easily accessible to producers, and they only need to build the oven, grind and sieve the charcoal, and construct the column for cachaça filtration. It was verified that efficiency of conversion of sugarcane bagasse into charcoal was 30%. The cost of these materials is low when compared with other copper-removal technologies. Moreover, the charcoal used in filtering can be reused as fertilizer because it contains the nutrients required for new plantations of sugarcane.

Conclusions

Selected yeast had similar cachaça production as compared to the Baker's yeast. The beverage produced

by fermentation using the selected yeast, contain a higher content of volatile acids in the distillate. The copper content in the beverage, in the experimental conditions used, was very high, with a maximum level of 25.16 mg L^{-1}. However, the charcoal obtained from sugarcane bagasse, from which sugarcane juice was extracted, can remove significant amounts of copper from the beverage to levels considered acceptable in the consumer market.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Use of a combined cultural-molecular method for isolation and identification of *Campylobacter* from broiler chicken in Morocco

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Campylobacters **are very important foodborne pathogens that raise the interest of food processors, researchers, as well as consumers and all stakeholders. Their contaminations can result in life threatening disorders, potentially leading to chronic sequelae such as Reiter's and Guillain-Barré syndromes or Crohn's Disease. Poultry has been identified as the main and most common reservoirs for *Campylobacter*. A survey was conducted in Morocco, from 2009 to 2012 to estimate the prevalence of *Campylobacter* in broiler chicken from about fifteen Moroccan cities that were most involved in the breeding of broilers. In this study, thermotolerant *Campylobacter* spp. were detected and identified from 165 samples by both cultural methods and molecular approaches based on polymerase chain reaction and 16S ribosomal RNA (rRNA) gene sequencing. The species were also genotyped by Restriction Fragment Length Polymorphism analysis. The conventional culture methods identified 97% of samples as positive for *Campylobacter* spp. The molecular approach based on 16S rRNA gene could not distinguish between *C. jejuni, C. coli and C. lari*. However, gyrB gene RFLP, allowed a good discrimination between the three species of *Campylobacter*. These results were also confirmed by Matrix Assisted Laser Desorption Ionization-Time of Flight and Mass Spectrometry proteomic profiling determination using libraries in the BioTyper 2.3 software. The present study identifies *C. jejuni* as the major source of contamination of poultry carcasses in Morocco.**

Key words: *Campylobacter*, chicken, *16S* rRNA gene*, gyrB gene,* sequencing, RFLP-PCR, MALDI-TOF MS.

INTRODUCTION

Campylobacteriosis is a zoonosis, renowned as the most frequently listed foodborne illness in humans at

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international level. The high incidence of campylobacteriosis as well as its duration and the possible after-effects make it highly important from a socio-economic perspective. The World Health Organization considers *Campylobacter* like the most common bacterial cause of human gastroenteritis in the world. (WHO, 2016). *Campylobacter* causes more cases of diarrhea compared to other foodborne pathogens like *Salmonella* or *Yersinia*, both in developing and developed countries (Humphries and Schuetz, 2015). In developing countries, *Campylobacter* infections among children under the age of two years as well as elderly and immunosuppressed individuals are particularly frequent, sometimes result in death (Samosornsuk et al., 2015). *Campylobacter* is reported as a causative agent of diarrhea among travellers staying repeatedly in developing areas including the Middle East, North Africa, Southeast Asia and Latin America (Kittitrakul et al., 2015). About seventeen (17) species, six (6) subspecies and four (4) biovars have been assigned to the *Campylobacter* genus, however, the most infectious species that has been frequently reported in humans are *C. jejuni* (subspecies *jejuni*) and, to a lesser extent, *C. coli*, and *C. lari*. Other species such as *C. hyointestinalis, C. upsaliensis,* and *C. sputorum* have also been isolated from patients with diarrheal disease, but are reported to be less frequent (Boelaert, 2016).

Campylobacter infection in humans may be due to the several reasons which include consumption or contact with undercooked poultry meat (Umaraw et al., 2017), cross-contamination from raw poultry, meat, foods that are in poor conservation conditions (potentially at low doses) or those which are commonly eaten without further heating (MacDonald et al., 2015). *Campylobacters* are readily detectable in the faeces of colonized birds, especially in chicken (Ishihara et al., 2016). In 2011, European Food Safety Authority (EFSA) has found that chickens and chicken meat might directly account for 20-30% of human cases (EFSA, 2011; Di Giannatale et al., 2016). As reported in the "Review of the poultry sector" of the FAO/UNO (Food and Agriculture Organization/United Nations Organization), in Morocco, the poultry sector constitutes one of the most dynamic agricultural fields where traditional poultry sector still provides local markets with white meats (13.5%) and broiler chicken is the most appreciated because it is an accessible source of meat to all social classes (Barkok, 2008).

Since its starting, at the beginning of the seventies, the intensive Moroccan avicolous sector developed in the absence of lawful measurements specific to this activity. However, since the nineties, the poultry industry has undergone a major expansion with the installation of a large number of livestock farms. The number of broiler farms has more than 5,000 units and many of them are located in the axis "*El Jadida - Casablanca – Kénitra*" due to the temperate climate of these cities, the proximity of the supply centers and marketing markets. Indeed, this axis covers 42% of broiler flocks on a national level. Practically all commercial breeding units of chicken in Morocco belong to the sector 2 of the FAO's classification. The semi- intensive farming or amateur (similar to the sector 3) are very rare. The marketing and the distribution of chicken in Morocco are done according to a primitive system; more than 90% of produced chickens are sold alive by small retailers. Those retailers supply either to markets or directly to farms. The consumer thus buys a live animal, and generally gives it then to any adjacent slaughter slaps for the service of sticking, plucking and evisceration. In Morocco, five commercial strains of exotic breeding chickens, called type "flesh", has been valued industrially, because of their rapid growth with an average of 2 ± 0.2 kg at an age of 40 ± 5 days: Ross, Hubbard, JV ("*Vedette*"), *Cobb* and *Arbor Acres*. *Ross*, *Hubbard* and *JV* strains hold 94 % of the national market. Ross would be in the lead because of its growth rate and its consumption index. Very few hatcheries specialize in breeding a single strain. Currently, the breeding of reproducers are very organized (Barkok, 2008).

The prevalence of *Campylobacter* infections, the species distribution in bacterial enteric infections and their antimicrobial resistance patterns of Morocco still remain unclear. This makes the detection of *Campylobacter* crucial in preventing its spread as well as in treating the disease or establishing the epidemiology of *Campylobacter* infection in Morocco.

The purpose of this work was to make a preliminary study of prevalence of *Campylobacter* in our country and to compare the accuracy of the classical and Molecular sub-typing methods for species identification among isolated *Campylobacter genus.*

MATERIALS AND METHODS

Ethics statement

The animals were kept and used in harmony with the instructions (policy, procedures and standards) of the Ethic Committee of the LRAM (*Laboratoire de Recherche et d'Analyses Médicales de la Fraternelle de la Gendarmerie Royale* – Morocco) which is responsible for monitoring internal researches involving the use of animals.

Sample collection

A 3-year study was performed from October 2009 to April 2012. One hundred and sixty five living broiler chickens were collected directly from commercial farms, retailers and markets of seventeen different sites.

We targeted fifteen Moroccan cities, which were mostly involved hatching using artificial incubators and the rearing of chickens for meat according to the review of the poultry sector of the FAO/UNO (Barkok, 2008). Namely: Rabat : from P1 at P30, Chichaoua : P31-P40, Settat : P41-P50, Tanger : P51-P55, Temara: P56- P65,

Figure 1. Sampling sites (The sampling sites in different geographical locations of Morocco are indicated by (black filled circle) on the map).

Nador: P66-P75, Agadir: P76-P85, Marrakech: P86-P95, El Jadida: P96-P105, Meknès: P106-P115 , Kénitra: P116-P125, Casablanca: P126-P135, Safi: P136-P145, Sefrou: P146-P155 and Kelâa Sraghna : P156-P165. The sampling sites of living chickens in different geographical locations of Morocco are indicated in Figure 1.

For our research works, we used five (5) strains of commercial reproductive broiler chickens such as Ross, Hubbard, JV ("Vedette"), Cobb and Arbor Acres without any distinction of strains or sex.

Chickens were chosen with an average body weight fixed at 2 ± 0.2 kg, aged about 8 weeks (40 ± 5 days). Most studies have shown that *Campylobacter* contaminates flocks of chicken between the second and the fourth week of breeding (Cean et al., 2015; Gonsalves et al., 2016; Rodgers et al., 2016) this has to be included in review.

The broiler chickens were transported separately in plastic boxes to the laboratory animal unit of the LRAM where the euthanasia of the broilers was carried out according to national laws and rules and internal policies, respecting the animal and preventing any cross-contamination.

The carcasses were scalded individually, with hot water, in the traditional way to reproduce the household gestures and to facilitate the process of plucking. To avoid the thermal shocks (where *Campylobacter* is very sensitive), carcasses were immediately directed to the laboratory for dissection and analyses. The carcasses were eviscerated and washed thoroughly with tap and warm water. The viscera (including intestines with their faeces) were removed aseptically and also the gallbladder to escape antibacterial activity of the bile (Begley et al., 2005; Merritt and Donaldson, 2009).

A total of 165 viscera samples from broilers were collected and placed into individual sterile plastic bags (Whirl-Pak Sample Bags, Nasco, B01323WA; VWR International), well-identified in a sterile bag, denoted P followed by a number, then they were weighed, mixed and homogenized thoroughly at 10,000 r.p.m. for 30 s in a

food crusher (Knife Mill Grindomix GM 200; Retsch).

Enrichment using selective liquid medium

The incorporation of enrichment procedures was to increase recovery of *Campylobacter* from our samples. All samples were serially diluted as per the standards (ISO 6887-1:1999), 25 g of viscera mix was weighed twice to get two subsamples in individual sterile stomacher bags (80015, bioMérieux). Each subsample was enriched in 225 ml of Preston Broth (PB) containing 5% (v/v) with Sheep Blood (Base Agar CM0689, supplement SR0204E; Oxoid) and incubated at 42°C for 18 h in a microaerophilic atmosphere using atmospheric generators and jars of incubation (GENbox Microaer, bioMérieux, France) with a microaerophilic gas mixture according to the ISO 10272 procedure, 1995.

Culture using selective agar

The detection of thermotolerant *Campylobacter* spp. (*C. coli, C. jejuni, C. helveticus, C. lari, C. sputorum, and C. upsaliensis*) by conventional bacterial culture method was conducted in accordance with the recommendations from the International Organization for Standardization (Standard Method ISO 10272 Procedures, 1995; 1996 et al., 1997).

Bacteria isolation

After incubation and for each subsample enriched PB, an aliquot of growth from an enrichment culture (initial suspension in tubes) was streaked onto Karmali Agar (Base Agar CM0935, supplement SR0205E; Oxoid) and modified Bützler Agar (Base Agar CM0271, supplement SR0085E; Oxoid). Following inoculation of sub-culture, all plates were incubated under microaerophilic condition using anaerobic jars gassed (GENbox Microaer, bioMérieux, France) at 42°C for 2 to 3 days depending on bacterial growth. After opening the jar and examining the cultures, if a second incubation was necessary, then new generators were employed.

Confirmation of thermotolerant *Campylobacter* spp

The identities of bacterial strains obtained from different samples were verified by microscopy and Gram staining. Standard phenotypic tests were used to identify species specific of field strains according to a previously described method (Wimalarathna et al., 2013). Strains were initially analyzed for the following properties: Gram negativity, spiral morphology, and microaerophilic growth dependency. Assays for oxidase and catalase activity as well as Hippurate hydrolysis were performed. Antimicrobial susceptibility tests with Nalidixic Acid and Cephalotin, were checked as described in ISO 10272:1995. Subsequently, Hydrogen Sulphide (H_2S) production was checked in TSI medium.

Bacterial strains for positive control

In this study, bacterial strains of some *Campylobacter* species were used as positive control in conventional PCR assays, as well as in quality control of productivity testing of media homemade:

i) *Campylobacter jejuni subsp jejuni (*ATCC 49943; AWEEL).
ii) *Campylobacter coli (*ATCC 43478; Microbiologics).
iii) *Campylobacter lari* (CIP 102722; kindly provided by Bacteriology Service of the French National Reference Center for *Campylobacter* and *Helicobacter*).

Molecular assays

DNA extraction

Bacterial DNA was extracted from colonies of 150 samples presumptive of *Campylobacter* spp. using the Sigma's GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, USA), according to manufacturer's instructions. Briefly, 1.5 mL of bacterial broth culture was pelleted at 12,000 to 16,000 × g for 2 min; cells were resuspended in 180 µL lysis solution A for Gram-negative bacteria or in 200 µL of lysozyme (200 units/ml) for Gram-positive bacteria. Then, 20 µL of Proteinase K was added to the cell suspension. After incubation at 55°C for 30 min, 200 µL of lysis solution C was added to the suspension. The suspended cells were then incubated at 55°C for 10 min. DNA was purified using GenElute Miniprep Binding Columns (Sigma-Aldrich, USA). DNA eluted out in sterile distilled water and stored at -20°C until use.

16S rRNA gene sequencing

The 16S rRNA gene sequence of 52 samples taken at random from 150 samples presumptive *Campylobacter* spp. was amplified using primers fD1 (5'-AGA GTT TGA TCC TGG CTC AG-3′) and rP2 (5′-AAG GAG GTG ATC CAG CC-3′), as described by Weisburg et al. (1991). PCR was carried out using 2.5 µl of 10X buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 0.4 µM of each primer, 1 U of Platinum Taq Polymerase (Invitrogen) and 5 µl (30 ng/µl) of template DNA in a 25 µl reaction volume under the following conditions : 4 min at 96°C (initial denaturation), 35 cycles of 10 s at 96°C (denaturation), 40 s at 52°C (annealing), 2 min at 72°C (extension), and one final step of 4 min at 72°C (extension cycle) employing the PCR thermocycler "Verity" (Applied Biosystems, Foster City). The amplified fragments were electrophoresed on 1% agarose gels and detected using ethidium bromide along with molecular weight markers. The PCR products were purified using EXOSAP-IT (USB, USA) and bidirectionally sequenced on an ABI 3130 Xl automated sequencer (Applied Biosystems, Foster City) using BigDye Terminator version 3.1 Kits using the same primers as of PCR. The analysis of electrophorogram was performed with the sequencing Analysis Software version 5.3.1 (Applied Biosystems, Foster City). The consensus sequences were edited and compared with published sequences available in GenBank, using BLAST tool of the NCBI.

RFLP analysis of gyrase B gene

For the gyrB gene amplification we used the universal primer mix described by Kawasaki et al. (Kawasaki et al., 2008) to amplify a 960-bp gyrB gene fragment from each *Campylobacter* reference strain and from each sample. A total of 150 samples were analysed by RFLP (Restriction Fragment Length Polymorphism).

The DNA template (1 µl) was amplified in a 50 µl reaction volume containing 1x PCR buffer, 2 mM $MgCl_2$, 0.25 U rTaq, (Sigma-Aldrich, USA) DNA polymerase, a 0.4 mM concentration of each of the four (4) dNTPs, and the universal primer mixture consisting of a 10 nM concentration of each primer. The cycling conditions consisted of an initial denaturation at 94°C for 5 min, followed by 45 cycles of denaturation (94°C for 30 s), annealing (55°C for 45 s), and extension (72°C for 30 s), with a final 7-min extension at 72°C. Computational prediction of the restriction fragment length analysis

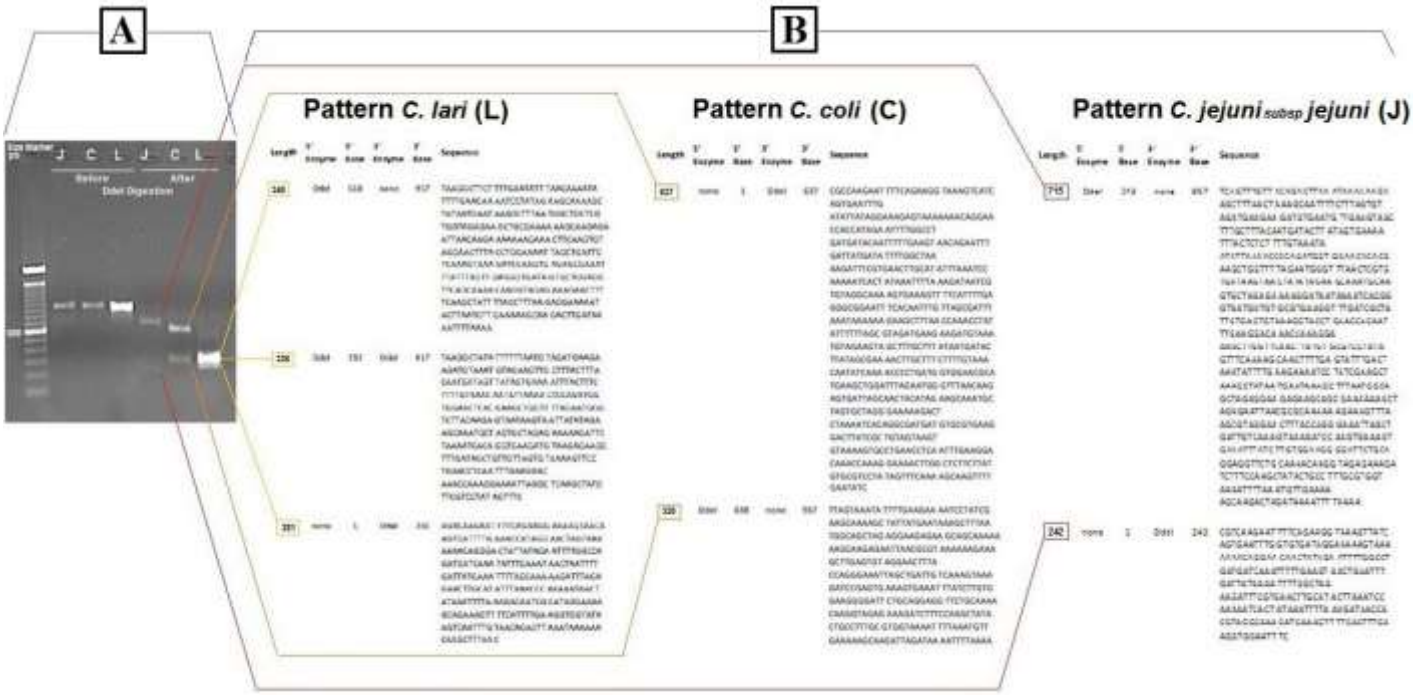

Figure 2. Validation of the gyrB PCR-RFLP approach. Pattern of gyrB PCR-RFLP after DdeI digestion (left, A) was compared to the computational prediction of the amplicons using the same restriction enzyme (right, B). The reference strains used were :(J: C. *jejuni* subsp *jejuni* (ATCC 49943); C: C. *coli* (ATCC 43478) and L: C. *lari* (CIP 102722). Lane MW, 100-bp molecular size markers.

of the 960-bp amplified region predicted that the DdeI enzymes would generate species-specific digestion patterns (http://www.restrictionmapper.org). For RFLP analysis, the purified PCR products were digested in a total volume of 20 µl with either 5 U of DdeI (Toyobo, Osaka, Japan). The resulting fragments were size separated using 2.5% Agarose prepared in 1x Tris-acetate-EDTA buffer and stained with Sybr green I dye (Invitrogen, Carlsbad, CA). The PCR-RFLP approach was validated by comparison of the DdeI I digestion pattern of the standard strains *C. jejuni*, ATCC 49943; C. *coli*, ATCC 43478 (Manassas, VA, USA) and *C. lari*, from Pasteur Institute Collection (CIP 102722) to the computational prediction of the amplicon–digestion using the same restriction enzyme as shown in Figure 2. All References *Campylobacter* species showed species-specific DdeI digestion patterns.

Mass spectrometry microbial identification system

The 155 strains isolated at LRAM were analyzed *in situ*. We used overnight bacterial cultures after their activation following Biomérieux's recommendations on incubation temperatures. For rapid identification of *Campylobacter* species, we worked on the IVD MALDI BioTyper system based on proteomic profiling and identification of bacteria directly from positive blood cultures samples using matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS).

Preparations of bacterial isolates for MALDI-TOF MS measurement were carried out as previously described (De Carolis et al., 2014; Rahi et al., 2016). Peptidic spectra were compared with the Bruker MALDI Biotyper library (version 5627) and software (version 2.3). Two distinct criteria were used to analyse the results of the spectral database search, a score value and a consistency category and Log score values range from 0.000 to 3.000 and are correlated with an explanation of genus and species consistency within the database. The software as highly probable species-level identification interprets a score ranging from 2.3 to 3.000. Log scores of between 2.00 and 2.299 represent secure genus identification and probable species-level identification.

RESULTS

In this study, thermotolerant *Campylobacter* strains isolated from different geographical areas of Morocco were identified with conventional methods, 16S rRNA gene sequencing, gyrB RFLP-PCR and MALDI-TOF MS determination methods.

Conventional method for the isolation and characterization of *Campylobacter* spp.

The conventional bacterial identification tests including Gram staining, culture and growth characteristics–and biochemical patterns showed that 160 out of the 165 samples (97%) were contaminated with thermotolerant *Campylobacter* strains whereas five (3%) samples were negative for *Campylobacter* spp. (samples: P11; P59;

Table 1. Conventional identification of isolates and distribution of species among *Campylobacter* positive samples.

Strain		Reference Method: Conventional Microbiology *(ISO 10272 : 1995 + 1996 + 1997)*													
		Test													
		Orientation			Genus Identification				Species thermotolerant Identification						
					TSI				Growth (°C)				Sensibility to antibiotics		
Number	%	Mobility	Gram	Catalase Oxidase	Glucose Saccharose	Lactose	Gas	H_2S	25	37	42	Hippurate	ANC	Cephalotin	Specimen
2	1.25	+++	-	+	-	-	-	-	-	+	+	+	S	R	*C. jejuni*
0	0	+++	-	+	-	-	-	-	-	+	+	-	S	R	*C. coli*
24	15	+++	-	+	-	-	-	-	-	+	+	-	R	R	*C. lari*
134	83.75	+++	-	+	-	-	-	-	-	+	+	+	R	R	*C.* spp

Abbreviations: Mobility +++ = very mobile; + = positive reaction, - = negative reaction; R = Resistance, S = Sensibility, ANC : Nalidixic Acid.

P67; P79; P149).

The bacterial identification of the 160 strains isolated from the samples revealed that 2 samples were positive in Hippurate hydrolysis test and sensitive to nalidixic acid and were identified as *C. jejuni.* About twenty four (24) samples were negative in Hippurate hydrolysis test and resistant to antibiotics which were recognized as *C. lari*. The remaining 134 samples identified as *Campylobacter* genus were unspecified because of their bacterial behaviour under conditions of Hippurate hydrolysis and antibiotics sensitivity reaction. These results are summarized in Table 1.

Detection and identification of *Campylobacter* species by molecular biology method (16S rRNA sequencing, PCR-RFLP of gyrB gene)

Out of the 165 samples, only 150 samples were analysed by molecular approaches because samples P1 to P10 were unable to be preserved and five samples were negative for *Campylobacter*.

16S rRNA gene sequencing

In this study the 16S rRNA gene were amplified for 105 samples from among the 150 samples. Sequencing of the 1540 bp 16S rRNA PCR fragments generated around 1200 to 1400-bp of sequence with an average Phred score of ≥ 20 (≥ 99% accurate). Sequences derived from the 16S rRNA genes of 105 presumptive *Campylobacter* strains were queried against NCBI's bacterial sequence databases using the web-based analysis tool. The 16S rRNA sequences queries returned a list of matching sequences that were a mixture of *C. jejuni, C. coli* and *C. lari* sequences, all possessing identity scores of 100-98%. This inability to differentiate between these three species using the commonly employed 16S rRNA sequencing procedure has been previously reported (Loong et al., 2016; Vondrakova et al., 2014) which prompted us to look for alternative methods.

PCR-RFLP of gyrB gene

Restriction enzyme analysis of PCR amplicons, known as PCR-RFLP, is a useful tool for molecular characterization of food-borne pathogens, including differentiation of thermophilic *Campylobacters* (Kamei et al., 2014). We have first validated this approach by using DNA from known *Campylobacter* reference strain. The results showed clearly that PCR-RFLP profiles matched perfectly with the computational prediction of the in sillico-digestion of the gyrB gene sequences of each reference strain.

Following the validation step, the approach was also applied to the samples. The results showed that out of the 150 strains positive for *Campylobacter* genus, 88 (59%) were identified as *C. jejuni*, 40 (27%) as *C. coli*, 2 (1%) contained both species *C. jejuni / C. coli.* A case of probable coexistence of two species in the same sample is shown for (P72 and P96) (Figure 3). None were

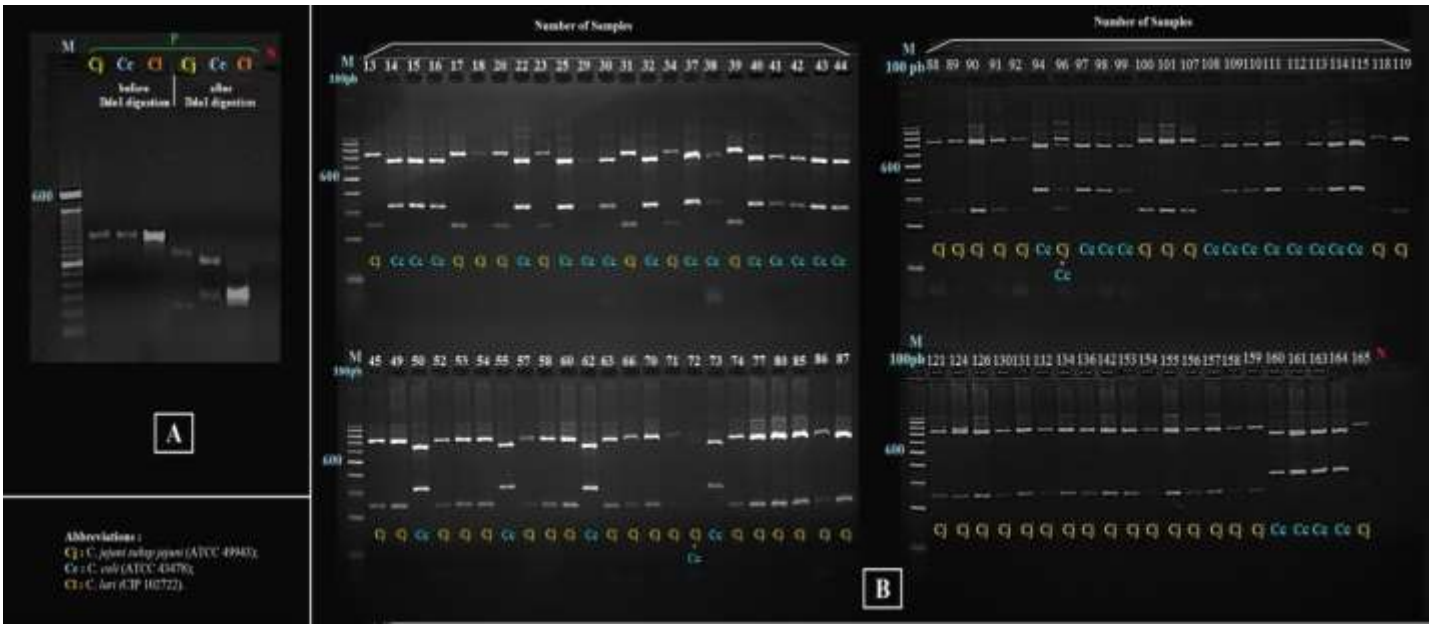

Figure 3. Illustration of gyrB RFLP profile. The RFLP profile obtained using Strain reference is shown in the left Panel (A). The right panel (B) shows the gyrB gene RFLP profile for some samples, after PCR amplification and Dde1 digestion. Lane M: molecular weight marker; N: negative control (no DNA); Cj (*Campylobacter jejuni* subsp *jejuni*); Cc (*Campylobacter coli*); and Cl (*Campylobacter lari*) (No C. *lari* was detected).

recognized as *C. lari* and the rest (20 samples): were unspecified because of unamplified DNA.

Identification of *Campylobacter* species by MALDI-TOF MS

Compared to the standard approach, the Matrix Associated Laser Desorption Ionization–Time Of Flight Mass Spectrometry (MALDI-TOF MS) seems to be interesting in terms of rapidity and cost (Branda et al., 2014). To assess the chosen methods and confirm the obtained results (by culture and molecular biology), we tested the 150 strains isolated from positive samples of *Campylobacter* genus by MALDI-TOF MS. Our result showed that among the 150 positive strains isolated. 111 (74%) were specified as *C. jejuni*, 39 (26%) as *C. coli* and (0%) as *C. lari*.

Comparison of the results obtained by the molecular approach based on gyrB PCR-RFLP to those obtained by MALDI-TOF MS

Using RFLP for gyrB gene, five samples P13; P27; P48; P61; P65 were identified as being *C. jejuni*, and three (3) samples were identified as *C. coli.* Moreover, the gyrB RFLP-PCR failed to identify the following 20 samples). Sample P21 was identified as *C. jejuni* by MALDI-TOF MS method. The other 19 samples which were unidentified by the molecular approach, the MALDI-TOF-MS method identified the P24; P47; P56; P64; P93; P102; P103; P104; P105; P106; P116; P120; P143; P162 samples as being *C. jejuni* while the samples P26; P33; P35; P51; P82 were identified as being *C. coli.* The results obtained from the different approaches has been summarised in Tables 2 and 3.

DISCUSSION

Campylobacter was considered for a long time as part of the normal microbiota of birds and the infection of broiler chickens had not previously been considered to cause disease (Ishihara et al., 2016). Challenging this paradigm, a recent work proved that *C. jejuni* is not merely a commensal in commercial broiler chickens but have significant influence on animal health and welfare in intensive poultry production. The incidence of campylobacteriosis cases among humans has been shown to correlate with the prevalence of *Campylobacter* spp. among broiler chickens (Bahrndorff et al., 2013) humans with gastroenteritis (Masanta et al., 2016).

Efficient and consistent techniques for isolation and identification of *Campylobacter* species in poultry are crucial to enable epidemiological and clinical

Table 2. A summary of *Campylobacters identification* estimated by various methods.

	Conventional Method				*Sequencing gyrB gene RFLP-PCR*					*MALDI-TOF MS*			
No. of samples analysed	**165**				**150**					**150**			
Result	**134**	**2**	**24**	**5**	**88**	**40**	**2**	**0**	**20**	**111**	**39**	**0**	**0**
	***C.* spp.**	***C. jejuni***	***C. lari***	**-**	***C. jejuni***	***C. coli***	***C. jejuni / C. coli***	***C. lari***	**-**	***C. jejuni***	***C. coli***	***C. lari***	**-**
%	***82***	***1***	***14***	***3***	***59***	***27***	***1***	***0***	***13***	***74***	***26***	***0***	***0***

Abbreviation: Specimen negative: -

Table 3. Concordant and discordant identification obtained by gyrB PCR-RFLP to MALDI-TOF MS.

Result	***Concordant identification***			***Discordant identification***				***Inconclusive (no result for one method)***	***Negative***	***Unknown unconserved***
				gyrB PCR-RFLP	***MALDI-TOF MS***	***gyrB PCR-RFLP***	***MALDI-TOF MS***			
	C. jejuni	***C. coli***	***C. lari***	***C. jejuni***	***C. coli***	***C. coli***	***C. jejuni***	***C.* spp**	**-**	***C.* spp**
Total	***80***	***23***	***0***	***11***		***17***		***19***	***5***	***10***
				28						
%	***48.5***	***14***	***0***	***17***				***11.5***	***3***	***6***

investigation. In this study, we have compared conventional and molecular methods to identify *Campylobacter* species in Moroccan poultry groups. Conventional methods based on morphological and biochemical proprieties are known to be relatively slow and laborious with a low discrimination power (Keramas et al., 2004). In our study also, 81% of the analysed samples (134/165 – Cf. Table 1), which were identified as *Campylobacter* genus, were unspecified because of their bacterial behaviour under conditions of antibiotics sensitivity reaction.

Biochemical tests carried out on strains whose morphological characteristics are suggestive of the genus *Campylobacter* make possible to identify the four major species of *Campylobacter* thermotolerants. Thus, the search for oxidase and catalase activity and the hippurate hydrolysis test are realized in association with the evaluation of sensitivity to two antibiotics, nalidixic acid and cephalotin. These tests are recommended by the ISO 10 272 standard (reference method for research thermotolerant *Campylobacter* in food). In practice, this biochemical identification of *Campylobacter* spp. presents difficulties of interpretation and some traps.

The hippurate hydrolysis test was widely used to distinguish thermotolerant *Campylobacter* species of *C. jejuni* (*C. jejuni* responds positively, *C. coli* and *C. lari* negatively), positive hippurate hydrolysis was pathognomonic to *C. jejuni* but the discovery of *C. jejuni subsp jejuni* strains negative to the hippurate test as *C. lari* strains (biotypes I and II) hippurate positive questions this method for the establishment of a formal distinction between the two species (Adzitey and Corry, 2011).

Also, *C. jejuni* strains resistant to nalidixic acid have appeared and pose problem for the use of antibiotic sensitivity as identification criteria (Wimalarathna et al., 2013).

As such, various methods based on nucleic acid amplification and sequencing has been proposed as an alternative to identify *Campylobacter* genus and the different species. These approaches determine specific thermophilic *Campylobacter* strains based on precise characterization of genomic DNA (Das et al., 2014) and included references.

In the context of studying bacterial phylogeny and taxonomy, the use of 16S rRNA gene sequences has been by far the most common housekeeping genetic marker employed for several reasons. These reasons include its

presence in almost all bacteria, often existing as a multigene family, or operons; the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more precise measure of time (evolution); and the 16S rRNA gene (1500-bp) is large enough for bioinformatics analysis (Vergis et al., 2013). However, it was shown that 16S rRNA analysis enables specific identification of most *Campylobacter* species. The exception was a lack of discrimination among the taxa *C. jejuni* and *C. coli* and atypical *C. lari* strains, which shared identical or nearly identical 16S rRNA sequences (Loong et al., 2016). Indeed, the results of this study also confirmed the inability of the 16S rRNA sequencing procedure to differentiate these three species. In fact, the BLAST based comparisons to NCBI's bacterial Sequence database provided a list of matching sequences that were a mixture of *C. jejuni, C. coli* and *C. lari* sequences, that possess identity scores of 100-98%. Knowing that suggested guidelines in literature describing the cut-off levels for sequence homology (percent identity) for proper identification of bacterial species ranging from ≥ 97% to ≥ 99% (Loong et al., 2016). Our results confirm the limitations of the 16S rRNA gene for resolving close relationship as previously demonstrated by Choi et al. (2016). The gyrB PCR-approach using DdeI as restriction enzyme provided a valuable tool for rapid and unambiguous identification of the majority of *Campylobacter* species. The analysis of our samples by gyrB PCR-RFLP showed a reliable discrimination between the three species in our collection. In fact, the 81% of our samples that failed to be characterized at species level with conventional methods have been well discriminated by gyrB PCR-RFLP. Nevertheless, the gyrB gene is not the only target gene with the power to identify pathogenic *Campylobacter* species. Other researches have shown that the lpxA and GTPase genes can also clearly identify and separate many of the *Campylobacter* species (Banowary et al., 2015; Klena et al., 2004).

With both methods, (gyrB RFLP-PCR and MALDI-TOF MS no strain was identified as *C. lari*. The misidentifications obtained with MALDI-TOF MS occurred only in samples where two strains coexisted. However, a discordance between gyrB RFLP-PCR and MALDI-TOF MS was found in 28 samples (Table 3). A total of 17 strains were identified as *C. coli* by gyrB RFLP-PCR and as *C. jejuni* by MALDI-TOF MS while 11 strains were specified as *C. jejuni* by gyrB RFLP-PCR and as *C. lari* by MALDI-TOF MS. For these strains, we have sequenced the gene encoding gyrB. All gyrB sequences of discordant strain when compared to the bacterial sequence database produced a collection of *Campylobacter* species sequences that were in perfect agreement with the results of the gyrB RFLP-PCR and that matched the query sequence with identity scores of 99-97%. However, the first match from the database that was in accordance with MALDI-TOF MS result had only a maximum identity of 90% (data not shown).

Conclusion

Overall, this study demonstrates that *Campylobacter* spp. viscera colonization in the broiler chicken samples collected all over Morocco had a high prevalence (97%) of natural contamination with *Campylobacter* spp. throughout the 3-year period compared to the other results found in other developed and developing countries. Our study revealed that *C. jejuni* was a more frequent pathogen than *C. coli and C. lari.*

This study may contribute to establishing proper control, monitoring and management strategies from the farm through to the consumer in order to reduce the incidence of campylobacteriosis.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Prevalence of IgG and IgM antibodies to Chikungunya virus among outpatients with febrile illness attending University of Maiduguri Teaching Hospital, Maiduguri, Borno State, Nigeria

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In Nigeria, there is paucity of information on the epidemiology of infections due to Chikungunya virus (CHIKV) especially among patients with febrile illness. Cases of febrile illness are usually associated with malaria and typhoid fever without considering the possibility of viral aetiology. This study was designed to determine the prevalence and identify other epidemiological parameters of CHIKV infections among outpatients with febrile illness attending University of Maiduguri Teaching Hospital, Nigeria. Sera from 370 patients were tested for presence of CHIKV immunoglobin (Ig) IgM and IgG antibodies using the enzyme-linked immunosorbent assay (ELISA). Of the 370 sera tested, 39 (10.5%) were positive for presence of CHIKV antibodies. A total of 24 (6.5%) tested positive for CHIKV IgM only, while none (0.0%) was positive for the presence of CHIKV IgG only. Fifteen (4.1%) of the serum samples simultaneously reacted to both IgG and IgM antibodies. A significant difference (p<0.0001) was observed in the distribution of CHIKV antibodies in relation to gender. The males had prevalence of 8.5% IgM antibodies as against 4.6% in females, 4.6% of females were positive for both CHIKV IgG and IgM antibodies, compared to 3.4% in males. The age group ≤60 years and the undisclosed age group were positive for the presence of CHIKV IgG and/or IgM antibodies. No significant difference was observed in the seasonal prevalence of CHIKV antibodies among the study subjects. Analysis of the prevalence of CHIKV antibodies in relation to clinical presentation in the patients revealed that headache and fever were the most frequently encountered ailments.

Key words: Chikunguya, antibodies, Borno.

INTRODUCTION

Chikungunya is derived from the root verb "kungunyala", meaning "to dry up" or "that which bends up" in reference to the stooped posture developed due to the rheumatological manifestations of the disease (Mohan et al., 2010). The disease is called "buka-buka" in Congo, meaning "broken-broken" reflecting the incapacitating arthralgias or to become contorted (Mohan et al., 2010). Chikungunya (CHIK) is caused by chikungunya virus

(CHIKV), formerly occurs only as an episodic arbovirus, is now a worldwide public health problem (Peyrefitte et al., 2007). This disease is gradually assuming a major health problem with potentially life-threatening and debilitating arthritis (Rashad et al., 2013). CHIKV is responsible for an acute infection with an abrupt onset of high fever, arthralgia, myalgia, headache and rash (Niyas et al., 2010; Yusof et al., 2011). Mothers afflicted with Chikungunya fever in the perinatal period can vertically transmit Chikungunya fever to neonates (Sebastian et al., 2009). Intrapartum transmission also contributes, while caesarean section does not appear to prevent the transmission. Neonatal Chikungunya fever is associated with fever, poor feeding, pain, distal edema, various skin manifestations, seizures, meningoencephalitis, and echocardiographic abnormalities in the newborn (Sebastian et al., 2009). The menace of this disease cuts across all ages and both sexes (World Health Organization, 2009). *Aedes* species (*Aedes aegypti, Aedes albopictus, Aedes africanus, Aedes furcifer, Aedes taylori* and *Aedes cordelliri*) are the principal vectors of CHIKV (Centre for Disease Control, 2012). Transmission cycle is from mosquito-to-man and man-to-mosquito; and such mosquitoes become infective approximately 10 days after feeding and remain infective for life (Centre for Disease Control, 2012). It is closely related to O'nyong-nyong (ONN) virus and believed to be enzootic throughout most of Africa and historical evidence indicates that it spreads to other parts of the world from this region (Powers et al., 2000). According to Weaver et al. (2012), monkeys are also involved in enzootic transmission cycles, where enzootic vector for example *A. furcifer* transmit CHIKV from monkeys to humans.

CHIKV is an emerging arbovirus that is widespread in tropical regions and is spreading rapidly to temperate climates with recent epidemics in Africa and Asia and also documented outbreaks in Europe and America (Rashad et al., 2013). In Nigeria, series of studies carried out gave rise to the evidence of CHIKV existence in the country (Moore et al., 1974; Fagbami and Fabiyi, 1975; Fagbami, 1977; Fagbami, 1978; Tomori et al., 1981; Adekolu-John and Fagbami, 1983; Adesina and Odelola, 1991; Baba et al., 2013; Ayorinde et al., 2016). Most of these previous studies on CHIKV infection conducted in Nigeria were carried out in South Western Nigeria. CHIKV has caused massive outbreaks in Africa and Asia and its magnitude and circulation especially in Nigeria remained poorly documented. Like most arboviruses, there is no specific surveillance carried out for CHIKV or viral screening for pyrexias of unknown origin in Nigeria. Therefore, there is paucity of information on the epidemiology of infections due to CHIKV especially among patients with febrile illness in Nigeria. Cases of febrile illness are usually associated with malaria and typhoid fever without considering the possibility of viral aetiology. This manuscript was designed to determine the prevalence and identify other epidemiological parameters of CHIKV infections among outpatients with febrile illness attending University of Maiduguri Teaching Hospital (UMTH).

MATERIALS AND METHODS

Study area

The study was conducted at the UMTH, Borno State, Nigeria. UMTH, a tertiary health institution designated as "Center of Excellence" in immunology and infectious diseases by the Federal Government of Nigeria. This institution has a 530 bed facility which is spread over 17 wards and serves a population of over 20 million in the North-Eastern sub-region of Nigeria as well as sizeable number across the borders of Cameroun, Chad and Niger Republics (Garba et al., 2011).

Study population

The target population were outpatients with febrile illness attending UMTH. Consent of the patients was sought orally and obtained before inclusion in the study. The ethical clearance for the study on patients was obtained from the Ethical Committee of UMTH, Borno State, Nigeria.

Sample collection

Blood samples were collected from a total of 370 febrile outpatients comprising 205 samples obtained during the rainy season (July, August and September 2012) and 165 samples collected during the dry harmattan season (November, December 2012 and January 2013). Using sterile syringes with needles, 5 ml of blood was collected from each patient into appropriately labeled sterile plain vacutainer tubes and kept at room temperature to clot. The sera from the clotted blood samples were separated by centrifugation at 134×g for 20 min. The harvested sera were stored in cryotubes at -20°C until tested. Socio-demographic information such as age, sex and clinical diagnosis were obtained from patients before blood sample collection.

Assay of serum samples for CHIKV antibody

Sera were analyzed for presence of IgM and IgG antibodies against CHIKV using the ELISA kits (IgM and IgG for human Chikungunya), manufactured by WKEA MED SUPPLIES CORP, Changchun 130012, China with LOT number 20130228. It is an indirect ELISA for specific detection of CHIKV IgM and IgG antibodies in human serum. Each serum sample was tested separately using the indirect ELISA employing secondary antibodies specific for IgM or IgG antibodies. In the indirect ELISA test, the sample antibody is sandwiched between the antigen coated on the plate and an enzyme-labeled, anti-species globulin conjugate. The addition of an enzyme substrate-chromogen reagent causes colour to develop.

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Table 1. Gender distribution of Chikungunya virus antibodies in human sera among outpatients with febrile illness attending UMTH, Borno State.

Total No. tested		Total No. positive	Distribution of antibodies in positive samples [No. (%) positive]		
			IgG only	IgM only	IgG + IgM
Female	194	18	0 (0.0)	9 (4.6)	9 (4.6)
Male	176	21	0 (0.0)	15 (8.5)	6 (3.4)
Total	370	39	0 (0.0)	24 (6.5)	15 (4.1)

χ^2=27.13, P<0.0001 (s).

Table 2. Age distribution of Chikungunya virus antibodies in human sera among outpatients with febrile illness attending UMTH, Borno State.

Age group	Total No. tested	Total No. (%) positive		
		IgG only	IgM only	IgG + IgM
0 – <15	63	0 (0.0)	5 (7.9)	4 (6.3)
15 – <30	136	0 (0.0)	5 (3.7)	7 (5.1)
30 – <45	55	0 (0.0)	4 (7.3)	0 (0.0)
45 – <60	28	0 (0.0)	1 (3.6)	1 (3.6)
60 – <75	3	0 (0.0)	0 (0.0)	0 (0.0)
75 – 90	2	0 (0.0)	0 (0.0)	0 (0.0)
Undisclosed age	83	0 (0.0)	9 (10.8)	3 (3.6)
Total	370	0 (0.0)	24 (6.5)	15 (4.1)

IgM only, χ^2= 5.042, df = 6, p= 0.5385 (ns); IgG+IgM, χ^2= 3.864, df = 6, p=0.6951 (ns).

This colour is directly proportional to the amount of bound sample antibody.

Statistical analysis

GraphPad Prism software was used for statistical analysis. Chi-square test was used to compare IgM and IgG antibodies to CHIKV in the study population. Fisher's exact test was used to compare the variables where appropriate.

RESULTS

Table 1 shows the gender distribution of CHIKV antibodies among outpatients with febrile illnesses attending UMTH, Maiduguri, Borno State. Out of the 370 sera tested, 39 (10.5%) were positive for presence of CHIKV antibodies. A total of 24 (6.5%) tested positive for CHIKV IgM only, while none (0.0%) was positive for presence of CHIKV IgG only. Fifteen (4.1%) of the serum samples simultaneously reacted to both CHIKV IgG and IgM and there was significant difference in the prevalence of IgG and IgM antibodies. A significant difference (p<0.0001) was observed in the distribution of CHIKV antibodies in relation to gender among outpatients with febrile illnesses attending UMTH, Maiduguri, Borno State. Males have significantly higher CHIKV antibodies than females in the study.

The distribution of CHIKV IgG and/or IgM antibodies among the age groups tested in the population under study, showed no significant difference (p>0.05). The age groups ≤60 years and the unclassified age group were positive for CHIKV IgG and/or IgM antibodies (Table 2). None of the samples from the age group >60 years was positive for CHIKV antibodies. This age group represents a very small minority of the overall population presenting with febrile illnesses in the study area.

The results of the present study showed no significant difference (p>0.05) in the prevalence of CHIKV antibodies between the rainy and dry seasons. The rainy season had prevalence of 16/205 (7.8%) for IgM only and 8/205 (3.9%) for IgG and IgM when compared with the dry season 8/165 (4.8%) for IgM only and 7/165 (4.2%) for IgG and IgM (Table 3).

No significant difference (p>0.05) was observed in the distribution of CHIKV antibodies in relation to clinical presentations (as observed by Clinicians) of the patients (Table 4).

However, considerable prevalence of antibodies was observed in patients with different ailments. For instance, head ache had 3/18 (16.7%) and 2/18 (11.1%) for IgM only and IgG and IgM, enteric fever/fever had 18/284 (6.3%) and 11/283 (3.9%) for IgM only and IgG and IgM, this is followed by malaria 2/9 (22.2%) and typhoid fever 1/36 (2.8%) for IgM only, abdominal pain ¼ (25.0%) and

Table 3. Seasonal distribution of Chikungunya virus antibodies in human sera among outpatients with febrile illness attending UMTH, Borno State.

Season	Total No. tested	Total No. (%) positive		
		IgG only	IgM only	IgG + IgM
Rainy [July – September]	205	0 (0.0)	16 (7.8)	8 (3.9)
Dry (Harmattan) [November to January]	165	0 (0.0)	8 (4.8)	7 (4.2)
Total	370	0 (0.0)	24 (6.5)	15 (4.1)

IgM only, p=0.2929 (ns); IgG + IgM, p=1.000 (ns).

Table 4. Distribution of Chikungunya virus antibodies from human sera in relation to clinical presentation (as observed by Clinicians) in patients attending UMTH, Borno State.

Clinical presentations	Total No. tested	Distribution of antibodies in positive sera		
		IgG only	IgM only	IgG + IgM
		No. (%) positive	No. (%) positive	No. (%) positive
Abdominal pain	4	0 (0.0)	0 (0.0)	1 (25)
Body/Joint pain	9	0 (0.0)	0 (0.0)	1 (11.1)
Dysentery	1	0 (0.0)	0 (0.0)	0 (0.0)
Enteric fever/fever	284	0 (0.0)	18 (6.3)	11 (3.9)
Fever + Headache	3	0 (0.0)	0 (0.0)	0 (0.0)
Fever + Abdominal pain	2	0 (0.0)	0 (0.0)	0 (0.0)
Fever + Joint pain	1	0 (0.0)	0 (0.0)	0 (0.0)
Headache	18	0 (0.0)	3 (16.7)	2(11.1)
Loss of stamina	2	0 (0.0)	0 (0.0)	0 (0.0)
Malaria	4	0 (0.0)	2 (50.0)	0 (0.0)
Weakness	6	0 (0.0)	0 (0.0)	0 (0.0)
Typhoid fever	36	0 (0.0)	1 (2.8)	0 (0.0)
Total	370	0 (0.0)	24 (6.5)	15 (4.1)

IgM only, χ^2=18.4, df=14, P=0.1893; IgG +IgM, χ^2 = 10.36, df=14, P=0.7358.

body/joint pains 1/9(11.1%) for IgG and IgM (Table 4).

DISCUSSION

Chikungunya is specifically a tropical disease that is relatively uncommon and poorly documented (Pialoux et al., 2007). The CHIKV, an emerging arthropod borne virus is widespread in tropical regions (Africa and Asia) and is spreading rapidly to temperate climates with recent outbreaks in Europe and the Americas (Rashad et al., 2013). The virus has increasingly great impact on man with potentially life-threatening and debilitating arthritis (Rashad et al., 2013). The 10.5% prevalence of CHIKV infections observed among outpatients with febrile illness, attending University of Maiduguri Teaching Hospital is similar to the reports previously obtained in Nigeria (Fagbami and Fabiyi, 1975; Fagbami, 1977; Adesina and Odelola, 1991). It was observed in this study that none of the sera tested was positive for IgG antibody only, but 4.1% were positive for both IgG and IgM, and 6.5% were positive for IgM only. IgM ELISA is the diagnostic test of choice for detecting recent infection and may be applied to single serum sample in some instances. The presence of IgG antibody indicates past infection. The prevalence of IgM antibody observed in this study is therefore an indication of a recent and active infection of CHIKV among the study population as vaccination against the disease has never been carried out in the study area. The absence of CHIK IgG only when compared with a relatively high prevalence of IgM only observed in this study could be an indication of the sporadic nature of CHIKV infection in the study area during the study period. In addition, the absence of CHIK IgG only is an indication that the CHIKV infection is not endemic during the study period. However, these observations contrasted with other studies (Sergon et al., 2008; Gerardin et al., 2008; Kumar et al., 2010; Sissoko et al., 2008 and Mohanty et al., 2013). The difference could be attributed to difference in geographical locations and periods of studies and could be attributed to the exposure of the population engaged in the rubber plantation to the infective biting of

Aedes albopictus as observed in previous studies. The present study was carried out in the Sudano-Sahelian vegetational zone which is a semi-arid area in north-eastern part of Nigeria where the vectoral activities of *A. albopictus* could be relatively low when compared with areas where the previous studies were carried out. The results of this study represent the first report on the prevalence of CHIKV in Sudano-Sahelian zone of Nigeria. The relatively high prevalence observed in study could be associated with low socio-economic condition, insurgency/terrorism and poor sanitation of the study area that could facilitate the abundance of *Aedes* vector species in the study area. A difference in gender prevalence of CHIKV infection was observed in the present study with the males showing higher prevalence rate than the females which is consistent with the observations made in previous studies (Suryawanshi et al., 2009; Kumar et al., 2010, 2011; Patil et al., 2013). This may be associated with the occupational risks engaged by the males. The males are usually engaged in occupations that may expose them to bites by the vectors. However, the present finding disagrees with the earlier reports (Balasubramaniam et al., 2011; Dwibedi et al., 2011; Mohanty et al., 2013). The difference may probably be as a result of difference in geographical location and presence of socio-economic factors that facilitated the breeding of *Aedes* vector in their study area.

It was observed in this study that the age group ≤60 years of age were found to be more susceptible to CHIKV infection which is in consonance with previous reports (Mohanty et al., 2013; Patil et al., 2013). This is the most active age group and coupled with movement of people outdoors during the day time when the activity of *A. albopictus* is at its peak, lesser personal protection and individual difference in immune response to diseases are some of the speculative reasons for increased susceptibility to CHIKV infection in this age group.

Seasonal prevalence of CHIKV infection among subjects in the present study revealed that CHIKV infection is more during rainy season (July to September) than dry season (November to January). This could be attributed to the increased vector abundance and activity during rainy season as observed in study and in previous investigations (Mavalankar et al., 2008; Suryawanshi et al., 2009; Balasubramaniam et al., 2011; Dwibed et al., 2011; Mohanty et al., 2013). The patients in the present study that exhibited fever and headache were observed to show the highest prevalence of CHIKV antibody which could be the major symptoms of CHIKV infection in this environment.

Conclusion

The results of this study on the prevalence of IgG and IgM antibodies to CHIKV among outpatients with febrile illnesses attending University of Teaching Hospital, Maiduguri, Borno State, has shown that out of the 370 sera tested, 39 (10.5%) were positive for the presence of CHIKV antibodies and 6.5 and 4.1% of the patients had only IgM and both IgG and IgM antibodies, respectively. This indicates recent infection and high prevalence of antibodies to CHIKV among the study population. The absence of IgG only antibody in the study population showed that the infection is not endemic but sporadic. It is possible to suggest that CHIKV infection is not endemic in the study area prior to this current study, due to the absence of CHIKV antibodies in the older age groups (≥60 years). The results of the study also showed that the gender, season and age as well as fever and headache are important factors influencing the prevalence of CHIKV antibodies in the study area. Comprehensive studies are needed to determine the seasonal distribution of CHIKV infection vis-à-vis vector dynamics and distribution. There is need to institute specific CHIKV/antibody surveillance and routine screening for the virus especially among patients with pyrexia and headache.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Full Length Research Paper

Aspergillus flavo furcatis: Aflatoxin test and milk-clotting protease production in submerged and solid state fermentation

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Proteolytic enzymes are metabolites that can be produced by microbial sources and develop important functions in food industry as in cheese manufacturing. However, it is necessary to ensure the final product safety by testing toxins production by microorganisms. As a result of this, the aim of this study was to investigate the production of proteases by *Aspergillus flavo furcatis* DPUA 1608 in submerged and solid state fermentation and also certify the non-production of aflatoxin by this species. The aflatoxin test was carried out using the method of ammonia vapor. In this test, *A. flavo furcatis* DPUA 1608 was inoculated in seven different media cultures (COA, YES, CZ, CYA, GMS, PMS and PDA) and the production of toxins was confirmed by the color change of culture reverse after adding a 25% (w/v) ammonia solution. The protease production was conducted using four inoculums (SAB+GLI, SAB+SAC, BDA+GLI and BDA+SAC) in three media cultures of submerged fermentation obtained by a base mineral solution (MA01, MAGli and MASac) and in solid state fermentation using açai seeds and rice bran as substrate (SAFA). According to ammonia vapor test, *A. flavo furcatis* is not an aflatoxin producer. There was no color change in the colonies reverse of any culture media. All crude extracts obtained in both fermentations were tested for protease production. The best protease activity was observed in the medium MA01 (inoculums SAB+SAC, BDA+GLI and BDA+SAC). Milk clotting activity was determined in all crude extracts of submerged and solid fermentation. However, the clot formed was considered as strong milk coagulation only in the media MA01 of submerged fermentation (inoculums SAB+SAC, BDA+GLI and BDA+SAC) and in all inoculums of solid state fermentation (SAFA). *A. flavo furcatis* DPUA 1608 showed potential milk-clotting protease production in both fermentations media used.

Key words: Ammonia vapor test, *Aspergillus*, milk-clotting enzymes, liquid fermentation, solid fermentation.

INTRODUCTION

Proteases are enzymes capable of hydrolyzing the peptide bond in a protein molecule. They represent one of the largest groups of industrial enzymes with increasing market demands due to their applications

in industrial process like in cheese manufacturing using rennet enzymes (Mandujano-González et al., 2016; Sandhya et al., 2005). Since the limited availability of mammalian rennet and most rennet of plants are unsuitable because they impart a bitter taste to cheese, microbial sources can be potential as substitute to animal rennet. Among the microbial sources, fungi as enzyme producers have many advantages, since they are normally generally regarded as safe (GRAS) strains and the produced enzymes are extracellular which makes its easy recuperation from fermentation broth (Ayana et al., 2015; Sandhya et al., 2005).

Aflatoxins are carcinogenic fungal metabolites commonly produced by some species of *Aspergillus* such as *Aspergillus flavus* and *Aspergillus parasiticus* (Blankson and Mill-Robertson, 2016; Kamika et al., 2016). These fungi can infect a number of foods and feeds such like peanuts and maize and cause contamination (Saito and Machida, 1999).

Aspergillus flavo furcatis is anamorphic fungi that have shown potential as enzyme producer (Teixeira et al., 2012). A recent study reported the production of milk-clotting enzymes by the species in submerged fermentation (Alecrim et al., 2015). On Czapek's solution agar, its colony spread rapidly, attaining a diameter of 6.0 to 7.0 cm in 10 to 12 days at room temperature (24-26°C) and showing dark olive-buff through brownish olive color when young, becoming sepia to mummy brown (Raper and Fennell, 1977).

Submerged fermentation (SmF) is a well-known process to produce enzymes by the growing of microorganisms in liquid substrate or in the presence of excess water while solid-state fermentation (SSF) involves the growth of microorganisms on solid substrates in the absence or near absence of free water in the space between particles (Bensmail et al., 2015; Singhania et al., 2010). Because of need for new microbial milk-clotting proteases sources due the shortage of rennet and the increase demand for cheese, the aim of this study was to investigate the protease production by *A. flavo furcatis* DPUA 1608 in submerged and solid state fermentation and also certify the non-production of aflatoxin by this species.

MATERIALS AND METHODS

Microorganisms

The culture of *A. flavo furcatis* DPUA 1608 (Culture Collection DPUA/Federal University of Amazonas-UFAM) was inoculated in test tubes containing (w/v): 1.0% sucrose, 1.0% meat peptone and 1.5% Agar and maintained at 30°C for seven days (Klich and Pitt, 1988).

Aflatoxin test by ammonia vapor

The aflatoxin test was carried out using the method reported by Saito and Machida (1999). The toxin production was confirmed by color change at colony reverse in the media used. Seven different media were used to determine the color change: Coconut (COA), yeast extract-sucrose (YES), Czapek (CZ), Czapek-yeast extract (CYA), glucose-mineral salts (GMS), peptone-mineral mix (PMS) and potato-dextrose agar (PDA). The strain (4 days growth) was inoculated at the center of solidified agar medium in Petri dishes and incubated at room temperature (25°C). To observe the color change of colony reverse, the dishes were placed upside down and a drop (0.2 ml) of 25% (w/v) ammonia solution was put into the lid of the Petri dish. The samples were prepared in triplicate.

Inoculum

Four different inoculums of *A. flavo furcatis* DPUA 1608 were used to select the best one after the fermentation processes. The strain was inoculated in test tubes containing: [1] glucose+meat peptone (SAB+GLI) [2] sucrose+meat peptone (SAB+SAC) [3] potato+glucose (BDA+GLI) and [4] potato+sucrose (BDA+SAC). The cultures were incubated at 30°C for 7 days and after this period, solutions of 10^5 mL^{-1} spores were prepared for use in each fermentation medium.

Fermentation media and culture conditions

The production of milk-clotting enzymes was made by submerged and solid state fermentation. The submerged fermentation was conducted by using three media cultures (MA01, MAGli and MASac) based on a mineral solution (MA01) ($g.L^{-1}$): [KH_2PO_4 (2.0); $(NH_4)SO_4$ (1.0); $MgSO_4.7H_2O$ (0.1); $Na_2HPO_4.7H_2O$ (0.9); yeast extract (1.0) and gelatin (5.0)]. In MAGli medium was added 1% (w/v) glucose in the mineral solution (MA01) and in MASac medium was added 1% (w/v) sucrose in the mineral solution (MA01) (Table 1). The fermentation was performed in 125 mL Erlenmeyer flasks containing 50 ml of sterilized medium with the spore solution (10^5 mL^{-1} spores) of all inoculums (SAB+GLI, SAB+SAC, BDA+GLI and BDA+SAC), incubated at 30°C, and 180 rpm for 72 h. The biomass was separated from the crude extract by vacuum filtration on Whatman filter paper number 1. The solid-state fermentation was carried out using two local agro industrial residues: açai seeds and rice bran (SAFA) [9:1]. The mixture (50 g, 60% humidity and pH 6.0) was distributed in 500 mL Erlenmeyer flasks and sterilized at 121°C for 15 min. After cooling, the flasks were inoculated using the same spore solutions (inoculums) of the submerged fermentation. The flasks were maintained at 30°C for 5 days. The enzymes were extracted by adding 250 mL of sterilized distilled water and submitted to agitation (180 rpm) for one hour. The biomass was separated from the crude extract by vacuum filtration on Whatman filter paper number 1.

Proteolytic activity assay

The proteolytic activity was determined according to the

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Table 1. Submerged fermentation media based on a mineral solution (MA01).

Medium	Medium composition (g.L $^{-1}$)
MA01	KH_2PO_4 (2.0); $(NH_4)SO_4$ (1.0); $MgSO_4.7H_2O$ (0.1); $Na_2HPO_4.7H_2O$ (0.9); Yeast extract (1.0), Gelatin (5.0)
MAGli	KH_2PO_4 (2.0); $(NH_4)SO_4$ (1.0); $MgSO_4.7H_2O$ (0.1); $Na_2HPO_4.7H_2O$ (0.9); Yeast extract (1.0), Gelatin (5.0), Glucose (10)
MASac	KH_2PO_4 (2.0); $(NH_4)SO_4$ (1.0); $MgSO_4.7H_2O$ (0.1); $Na_2HPO_4.7H_2O$ (0.9); Yeast extract (1.0), Gelatin (5.0), Sucrose (10)

MA01 = Mineral soution base medium; MAGli = Mineral solution base medium+glucose; MASac = Mineral solution base medium+sucrose.

method described by Leighton et al. (1973). The crude extracts (0.15 mL) were mixed with 1.0% (w/v) azocasein (0.25 mL) in 0.2 M Tris-HCl buffer, pH 7.0. The incubation was made in absence of light for one hour. The reaction was stopped by adding 1.2 mL of 10% (w/v) tricloroacetic acid (TCA), centrifuged at 10 000 rpm and the supernatant (0.8 mL) was transferred to 1.4 mL of 1 M NaOH. One unit of proteolytic enzyme was defined as the amount of enzyme that produces a 0.1 increase of absorbance in 1 h at 440 nm. All samples were prepared in triplicate.

Milk-clotting protease assay

Milk-clotting activity was determined according to Alecrim et al. (2015) using 10% (w/v) skimmed milk powder in 0.05 M $CaCl_2$, as substrate. The milk solution (5 mL) were distributed in test tubes and pre-incubated in water bath at 50°C for 15 min. The enzyme extract (0.5 mL) was added to the milk solution and the clot formation was observed while manually rotating the test tube. The time, in seconds, at which the first particles were formed was measured. All samples were prepared in triplicate.

The milk-clotting activity unit (U) was defined as the amount of enzyme required to coagulate 1 mL of substrate in 40 min at 50°C. Milk-clotting activity (U) (Equation 1) and the ratio (R) (Equation 2) were calculated using the following equations:

$$U = \frac{2400 \text{ x } S}{T \text{ x } E} \quad (1)$$

$$R = \frac{\text{Milk} - \text{clotting activity}}{\text{Proteolytic activity}} \quad (2)$$

Where, 2400 is the total time of milk-clotting activity (s), S is the milk volume (mL), E is the enzyme volume (mL) and T is the time of clotting formation (s). The samples were grouped into two classes according to the formation of compact milk clot and milk whey separation in the test tube: strong milk coagulation (distinct clot and abundant whey) and weak milk coagulation (clot formation without clear separation of the whey).

Statistical analysis

In all experiments, the data were subjected to descriptive statistical analysis of variance and the averages were compared by Tukey's test ($p<0.05$) using Minitab program, version 17.0. (Minitab, 2010).

RESULTS AND DISCUSSION

Aflatoxin test by ammonia vapor

A. flavo furcatis DPUA 1608 did not produce aflatoxin in any of the media tested (Figure 1). In the colony reverse, the change of color induced by ammonia vapor was not observed. Saito and Machida (1999) observed the change of color in 13 strains of *A. parasiticus* and in 55 strains from 83 of *A. flavus* while the strains of *A. oryzae* (19 strains) and *Aspergillus sojae* (5 strains) did not produce aflatoxin.

Aflatoxins have been studied because of the possible hazards to human health. They commonly are produced by *A. flavus* and *A. parasiticus*. In the study of Kulkarni and Chavan (2015), nine of twelve isolates of *A. flavus* (three maize varieties), showed the aflatoxin production represented (75%) by thin layer chromatography (TLC) method. However, in the same study, the aflatoxin production was also detected by the ammonia vapor test. In this test, only one isolate was found to be non-toxigenic.

Nair et al. (2014) also reported aflatoxin production by the ammonia vapor test in *Aspergillus* strains isolated from spice samples that previously were considered positive by HPLC analysis. The ammonia vapor test was also effective and considered easy and not expensive in the study of Zrari (2013). The author isolated strains of *Aspergillus* spp. showed both aflatoxigenic and non-aflatoxigenic classifications.

The result of the present study in respect to *A. flavo furcatis* DPUA 1608 is important due the intention to implicate the enzymes of this fungus in food industry. Another test might be done to consider it as safe or GRAS as citotoxic tests *in vitro* (*Artemia salina* and hemolysis, for example).

"GRAS" is an acronym for the phrase generally recognized as safe. Under sections 201(s) and 409 of the Federal Food, Drug, and Cosmetic Act (the Act), any substance that is intentionally added to food is a food additive, that is subject to premarket review and approval by FDA, unless the substance is generally recognized, among qualified experts, as having been adequately shown to be safe under the conditions of its intended use, or unless the use of the substance is otherwise excluded from the definition of a food additive (FDA, 2016).

Protease production and milk-clotting activity

In this study, *A. flavo furcatis* synthesized and excreted

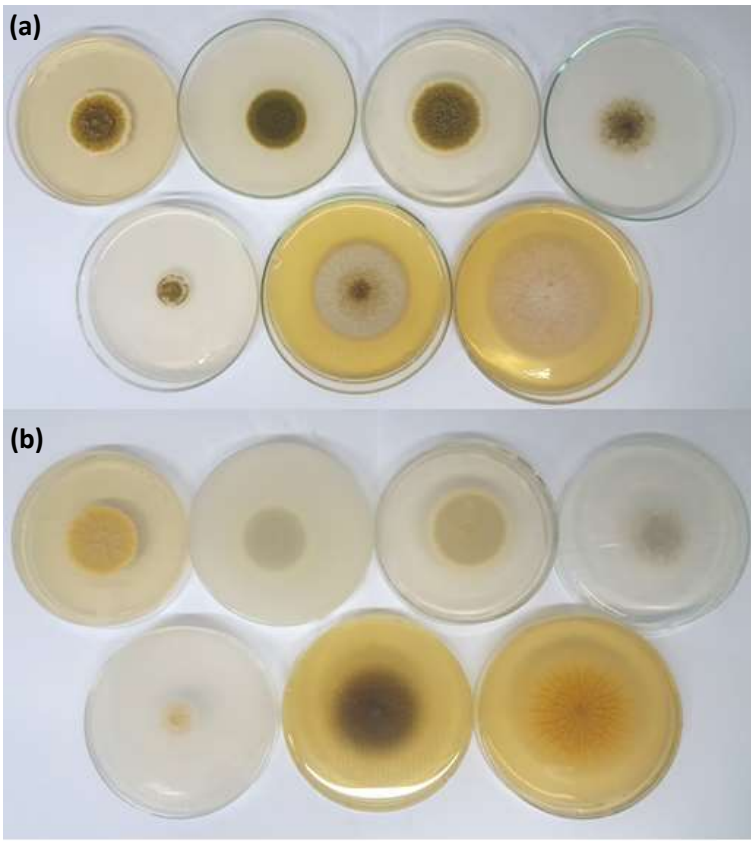

Figure 1. Results of aflatoxin production by ammonia vapor test. Front (a) and reverse (b) of *A. flavo furcatis* in BDA, COA, CYA, CZ, GMS, PMS and YES plates, respectively (from the top to the bottom).

proteases from all media of submerged and solid-state fermentation (Table 2). Comparing the media and inoculums used in submerged fermentation, the proteolytic activity was higher in the media supported with mineral solution base (MA01) in the inoculums SAB+SAC, BDA+SAC and BDA+GLI (53.00, 52.40 and 50. 66 U/mL, respectively). In the inoculum supported with SAB+GLI, the activity was 0.49 U/mL. In the other two media of submerged fermentation (MAGli and MASAc), the activity was low in all inoculums. In MAGLi, the protease activity were 0.75, 0.75, 0.88 and 0.93 U/mL (SAB+GLI, SAB+SAC, BDA+GLI and BDA+SAC, respectively) and in MASAc, the proteases activity were 0.67, 0.84, 0.71 and 1.53 U/mL (SAB+GLI, SAB+SAC, BDA+GLI and BDA+SAC, respectively). In the media of solid fermentation, the proteolitic activity was determined in all crude extracts from the inoculums used: SAB+GLI, SAB+SAC, BDA+GLI and BDA+SAC (17.31, 16.97, 13.27 and 13.60 U/mL, respectively).

The milk-clotting activity was determined in all crude extracts for the submerged and solid fermentation. However, in the submerged fermentation, according to the classification of coagulation established, the milk strong coagulation was observed only in the mineral solution base media (MA01) (Figure 2A) (inoculums SAB+SAC, BDA+GLI and BDA+SAC). The other crude extracts of submerged fermentation (MAGli and MASac) also promoted milk clotting activity but it was considered as weak milk coagulation (Figure 2B). In the solid-state fermentation, the milk coagulation was observed in all crude extracts using açai seeds and rice bran (inoculums SAB+GLI, SAB+SAC, BDA+GLI and BDA+SAC).

Alecrim et al. (2015) observed similar results of milk coagulation using the same strain in submerged fermentation with agro industrial residues as substrates. The authors reported three classifications of milk-clotting: strong milk coagulation, weak milk coagulation and milk without coagulation. The statistical analysis of milk-clotting activity and coagulant ratio were made only with the results of the crude extracts (submerged and solid fermentation) that showed milk strong coagulation in the visual classification: MA01 (Inoculums SAB+SAC, BDA+GLI and BDA+SAC) and SAFA (Inoculums SAB+GLI, SAB+SAC, BDA+GLI and BDA+SAC).

Table 2. Protease activity (U/mL), milk-clotting activity (U), coagulant ratio (R) and clot classification of *A. flavo furcatis* DPUA 1608 enzymes produced in submerged and solid-state fermentation.

Media / Inoculum		Proteolytic activity (U/mL)	Milk-clotting activity (U)	Coagulant Ratio (R)	Clot Classification
Submerged fermentation	MA01 (SAB+GLI)	0.49±0.10^{f}	45.80	93.7	Weak
	MA01 (SAB+SAC)	53.00±0.07^{a}	470.71±9.23^{c}	8.90±0.20^{e}	Strong
	MA01 (BDA+GLI)	50.66±0.11	507.09±6.14^{b}	10.03±0.11^{d,e}	Strong
	MA01 (BDA+SAC)	52.40±0.35^{a}	595.12±8.45^{a}	11.33±0.21^{d}	Strong
	MAGli (SAB+GLI)	0.75±0.04^{e,f}	122.11	161.60	Weak
	MAGli (SAB+SAC)	0.76±0.75^{e,f}	107.35	142.10	Weak
	MAGli (BDA+GLI)	0.89±0.07^{e,f}	199.21	224.10	Weak
	MAGli (BDA+SAC)	0.93±0.00^{e,f}	188.25	201.70	Weak
	MASac (SAB+GLI)	0.67±0.00^{e,f}	110.31	165.50	Weak
	MASac (SAB+SAC)	0.84±0.07^{e,f}	195.95	232.00	Weak
	MASac (BDA+GLI)	0.71±0.03^{e,f}	192.80	271.10	Weak
	MASac (BDA+SAC)	1.54±0.11^{e}	254.17	165.80	Weak
Solid-state fermentation	SAFA (SAB+GLI)	17.31±0.19^{c}	389.70±17.8^{e}	22.53±0.93^{b}	Strong
	SAFA (SAB+SAC)	16.98±0.27^{c}	428.66±7.66^{d}	25.27±0.59^{a}	Strong
	SAFA (BDA+GLI)	13.27±1.04^{d}	247.63±8.67^{f}	18.70±0.87^{c}	Strong
	SAFA (BDA+SAC)	13.60±0.35^{d}	273.00±10.5^{f}	20.07±0.66^{c}	Strong

Means followed by the same letters in the columns did not differ from one another by the Tukey's test ($p < 0.05$)/ (±) = st. dev./ DPUA= Code of Culture Collection from Federal University of Amazonas/MA01 = Mineral solution base medium; MAGli = Mineral solution base medium+glucose; MASac = Mineral solution base medium+sucrose; SAFA= Açai seeds+rice bran/ Inoculums: SAB+GLI= Glucose+Peptone; SAB+SAC= Sucrose+Peptone; BDA+GLI= Potato+Glucose; BDA+SAC= Potato+Sucrose; NMCA= No milk-clotting activity.

Figure 2. Classification of the samples according to clot and whey formation: (A) Strong milk coagulation (B) Weak milk coagulation.

The milk-clotting activity of *A. flavo furcatis* crude extracts were more significant in the submerged fermentation as compared to the solid state fermentation. From the inoculums used in the submerged fermentation, the most significant milk-clotting activity was BDA+SAC (595.12 U) followed by BDA+GLI and SAB+SAC (507.09 and 470.71 U, respectively). In the solid-state fermentation, the most significant inoculums to milk-clotting activity was SAB+SAC (428.66 U), followed by SAB+GLI, BDA+SAC and BDA+GLI (389.70 U, 273.00 U and 247.63 U) (Table 2).

Ayana et al. (2015) reported the production of milk-clotting enzymes by *Mucor mucedo* in liquid medium containing glucose, peptone, casein, KHPO, olive cake and corn steep liquor. *Aspergillus tamari* and *Penicillium pinophillum* also demonstrated great milk-clotting protease production in liquid medium containing whey as one of the main substrates (Benlounissi et al., 2014). In the study of Bensmail et al. (2015), *A. niger* produced proteolytic enzymes with wheat bran as substrate in submerged and solid-state fermentation. Castro et al. (2014) also studied milk-clotting enzymes secreted by *A. niger*. The authors used wheat bran, soybean meal and cottonseed meal as substrates in solid-state fermentation.

Solid state fermentation an appropriate method

because solid substrates resemble the natural habitat of fungi improving their growth and enzymes production. The simplicity, low cost, high yields and concentrations of the enzyme sand are some of the advantages of this process (Castro et al., 2014).

However, in submerged fermentation, the conditions are monitored with greater accuracy as compared to solid state fermentation. Almost all the large-scale enzyme producing facilities are using the proven technology of submerged fermentation due to better monitoring and ease of handling (Singhania et al., 2010). Both submerged and solid-state fermentation are suitable for use of inexpensive and widely agricultural residues as substrates (Sumantha et al., 2006)

According to coagulant ratio (R), there was significant difference between the inoculums used on submerged fermentation and solid state fermentation. In the solid state fermentation, the most significant value (25.27) was observed in the inoculums supported with SAB+SAC followed by SAB+GLI, BDA+SAC and BDA+GLI (22.53, 20.06 and 18.70, respectively) (Table 2). In the submerged fermentation inoculums, the highest value of coagulant ratio was observed in BDA+SAC (11.33), followed by BDA+GLI (10.03) and SAB+SAC (8.90).

The coagulant ratio (R) demonstrates the potential commercial suitability of milk-clotting enzyme in cheese manufacturing.

Enzymatic preparations used for milk clotting usually exhibit proteolytic action, but is important that the enzymes have specificity of cleavage (Phe_{105}-$Met1_{06}$ connection of casein). It defines a good coagulant (Perry, 2004; Visser, 1993; Barros et al., 2001; Hashem, 1999; Merheb-Dini et al., 2010; Yegin et al., 2011).

Conclusions

The strain of *A. flavo furcatis* does not produce aflatoxin in the media tested by the ammonia vapor method. The color change in the reverse of colonies was not observed. Proteolytic milk-cloltting enzymes were produced by *A. flavo furcatis* in submerged and solid fermentation media. In submerged fermentation, MA01 was the medium that promoted the highest activity of these enzymes in three of the inoculums used. In solid fermentation, açai seeds and rice bran showed potential as substrates to produce proteolytic milk-clotting enzymes by the microorganism.

The coagulant ratio was higher in crude extracts from solid fermentation than the submerged fermentation. The inoculums used in the process promoted the production of milk-clotting proteases. In submerged fermentation, the best inoculum was BDA+SAC (MA01) and in solid fermentation, the best inoculum was SAB+SAC.

The properties of *A. flavo furcatis* enzymes in this study encourage future milk-clotting characterization and cheese production experiments to check its potential as microbial coagulant source.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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