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Full Length Research Paper

Prevalence and molecular typing of extended-spectrum β-lactamases in *Escherichia coli, Enterobacter cloacae and Citrobacter freundii* isolates from Laghouat Hospital, Algeria

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The antibiotic resistance of enterobacteriacae knows a worldwide worrying evolution with an increase of the extended-spectrum β-lactamases. The present study was to determine the prevalence and molecular typing of extended-spectrum β-lactamases (ESBLs) in clinical isolates of *Escherichia coli, Enterobacter cloacae,* and *Citrobacter freundii*, isolated between January 2010 and December 2012, at the Laghouat "Ahemida Ben Adjila" hospital, Algeria. Antimicrobial susceptibility testing was determined by disk diffusion on Mueller Hinton agar. Genetic transfers were performed by conjugation and plasmid DNA was extracted by the alcalin-lysis method. The characterization of ESBL genes were examined using PCR amplification and DNA sequencing and the clonal relatedness was investigated by ERIC-PCR. During the study period, twenty-one (8.23%) isolates were found to produce ESBLs, distributed as follows: 13 isolates of *E. coli* (61.9%), 6 isolates of *E. cloacae* (28.57%) and 2 isolates of *C. freundii* with 9.52 %. The CTX-M-15 ESBL were predominant (95.24%), followed by TEM-4 (14.28%) and SHV-12 (4.76%). ERIC-PCR analysis showed that the isolates are genetically unrelated and conjugation experiments showed that *bla*$_{CTX-M-15}$ gene was transferred on a conjugative plasmid of high molecular weight (≈130 kb). This study indicated a high prevalence of CTX-M-15 enzymes among *E. coli, E. cloacae* and *C. freundii* in Laghouat hospital, Algeria.

Key words: Multiresistant bacteria, extended spectrum β-lactamase, CTX-M-15, genotyping, Algeria.

INTRODUCTION

ESBL-producing bacteria are responsible for many local, national and international outbreaks which originated from the different hospital wards and mostly in intensive care units (Rodriguez-Villalobos and Struelens, 2006). Infections due by these strains have been associated with high mortality in affected patients, and represent an

increased risk of therapeutic failure and are associated with longer duration of hospital stay and higher hospital charges (Soraas et al., 2013).

Production of extended-spectrum β-lactamases (ESBLs) is the principal mechanism of resistance to oxyimino-cephalosporins evolved by members of the family Enterobacteriaceae (Marco et al., 2013). These enzymes can hydrolyse penicillins, first, second and third-generation cephalosporins, and aztreonam, but do not hydrolyse cephamycins or carbapenems (Chanal et al., 1992). The activity of ESBL can be inhibited by β-lactamase inhibitors such as clavulanic acid (Paterson, 2000). This family of plasmid-mediated ESBL belongs to Ambler class A and group 2be of the Bush-Jacoby and Medeiros classification.

In the recent years, a new family of plasmid-mediated ESBLs called CTX-M which preferentially hydrolyze cefotaxime has emerged. CTX-M enzymes are not closely related to TEM or SHV β-lactamases, as they only show approximately 40% similarity in sequence (Shahid et al., 2009). These ESBLs have been classified into five phylogenetic families on the basis of their amino acid identities: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTXM-25 (Bradford, 2001; Bonnet, 2004).

CTX-M genes might be associated with insertion-sequence ISEcp1 or related insertion sequences involved in their expression and mobilization (Poirel et al., 2003). It is encoded by transferable plasmids that vary in size from 7 to 160 kb (Bonnet, 2004).

Now, CTX-M-15 β-lactamase, which was first described in 2001, it is recognized as the most widely distributed CTX-M enzyme (Livermore et al., 2007). This β-lactamase is found mainly in Enterobacteriaceae and was recently named “plasmids of resistance responsible for outbreak” because of their capacity to acquire genes of resistance and to transfer among bacteria (Coque et al., 2008).

The majority of previous studies on ESBL-producing isolates have been reported at the west, centre and east of Algeria (Baba Ahmed-KaziTani et al., 2013; Ramdani-Bouguessa et al., 2006; Gharout-Sait et al., 2012). But no study has been performed at southern regions of Algeria.

In this context, the aim of this study was to determine the prevalence and molecular typing of extended-spectrum β-lactamases (ESBLs) producing clinical isolates of *Escherichia coli, Enterobacter cloacae,* and *Citrobacter freundii* isolated in the Laghouat "Ahemida Ben Adjila" hospital, Algeria.

MATERIALS AND METHODS

Clinical isolates

During a three years period from January 2010 to December 2012, 255 non-repetitive clinical isolates of *E. coli*, *E. cloacae* and *C. freundii* were isolated from patients hospitalized at the Laghouat "Ahemida Ben Adjila" Hospital, Algeria. These isolates were obtained from various clinical specimens (including urine, pus, blood-culture, catheters and rectal swabs), and only one isolate per patient was investigated. The samples were performed on hospitalized patients from different wards: General surgery, orthopedics, women medicine, men medicine, pulmonology, obstetrics-gynecology, pediatrics ward and intensive care unit. All clinical isolates of enterobacteria were identified with the API 20E® system (bioMérieux, Marcy l’Etoile, France).

Antimicrobial susceptibility testing and ESBL detection

The susceptibility to 32 antibiotics was determined by the standard disk diffusion method on Mueller-Hinton agar recommended by the Antibiogram Committee of the French Society for Microbiology (CASFM, 2010).

The following antibiotics (Oxoid, England) were used: Amoxicillin (25 µg), amoxicillin/clavulanic acid (30 µg), ticarcillin (75 µg), ticarcilline/clavulanic acid (85 µg), piperacillin (75 µg), piperacillin + tazobactam (85 µg), cephalotin (30 µg), cefuroxime (30 µg), cefixime (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), cefepime (30 µg), cefpirome (30 µg), imipenem (10 µg), aztreonam (30 µg), cefoxitin (30 µg), gentamicin (15 µg), tobramycin (10 µg), amikacin (30 µg), nalidixic acid (30 µg), ofloxacin (5 µg), ciprofloxacin (5 µg), kanamycin (30 µg), fosfomycin (50 µg), tetracycline (30 µg), chloramphenicol (30 µg), sulfonamide (200 µg), netilmicin (30 µg), trimethoprim (5 µg), trimethoprim/sulfamethoxazole (25 µg), colistin (50 µg), and ceftazidime/clavulanic acid (30/10 µg).

Extended spectrum β-lactamase (ESBL) production was phenotypically confirmed using the double-disk synergy test, described by Jarlier et al. (1988). Synergy was determined between a disc of amoxicillin-clavulanate (20/10 µg) and a 30-µg disc of each third-generation cephalosporin test antibiotic (cefotaxime, ceftriaxone, ceftazidime, and aztreonam) placed at a distance of 20 mm from center to center on a Mueller-Hinton Agar (MHA) plate swabbed with the test isolate. Clear extension of the edge of the inhibition zone of cephalosporin toward the augmentin disc was interpreted as positive for ESBL production (Bradford, 2001; Giriyapur et al., 2011). *E. coli* ATCC 25922 was used as quality control strains.

Isoelectric focusing (IEF)

The β-lactamases isolated from clinical isolates were characterized by isoelectric focusing (IEF) according to the protocol determined by Bonnet and Coll (2000). IEF of β-lactamases was performed with polyacrylamide gels containing Ampholines with a pH range of 3.5 to 10. Thus, the following β-lactamases: CTX-M-1 (pI 8.4), CTX-M-14 (pI 7.9), CTX-M-15 (pI 8.6), TEM-3 (pI 6.3) and SHV-2 (pI 7.6) were used as the reference bands of known β-lactamases.

Extraction of bacterial DNA

Total DNA of ESBL-producing isolates was extracted by boiling to100°C for 10 min a suspension of the strains in 200 µl of distilled water and centrifugation for 7 min at 13,000 × g, then the supernatant obtained was stored at -20°C. PCR experiments were performed with these crude lysates.

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Table 1. Primers used in PCR for detection of *bla*- genes.

Target	Primer	Sequence	Annealing temperatures (°C)	References
TEM	TEM A TEM B	5' - TAA AAT TCT TGA AGA CG - 3' 5' - TTA CCA ATG CTT AAT CA - 3'	44	Heritage et al. (2001)
SHV	SHV 105q SHV 149p	5' - TTA GCG TTG CCA GTG CTC GAT - 3' 5' - CGC TTC TTT ACT CGC CTT TAT - 3'	54	Rasheed et al. (1997)
CTX-M-1	CTXM1 A2 CTXM1 B2	5' - CTT CCA GAA TAA GGA ATC - 3' 5' - CCG TTT CCG CTA TTA CAA - 3'	48	De Champs et al. (2004)
ERIC–PCR	ERIC2	5' - AAG TAA GTGACT GGG GTG AGC G - 3'	64	Dumarche et al. (2002)

Molecular characterization of *bla* genes

Genes encoding for extended-spectrum β-lactamases: bla_{CTX-M}, bla_{SHV}, bla_{TEM} were identified by PCR method (Polymerase Chain Reaction) (De Champs et al., 2004).

Primers used to amplify β-lactamases genes and annealing temperatures are shown in Table 1, thus the operating conditions and assay methods were performed to all ESBL producing isolates as previously described in detail (Lagha et al., 2014). The PCR products were visualized using UV after migration in agarose gel 1% and staining with ethidium bromide.

DNA sequencing was performed with the dideoxy chain termination method (Sanger et al., 1977), in GATC Biotech AG (European Custom Sequencing Centre, Gottfried-Hagen-Stra ße 20, 51105 Köln). The nucleotide and deduced protein sequences were analyzed using the Codon Code Aligner software and compared to sequences available at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov).

Enterobacterial repetitive intergenic consensus PCR (ERIC-PCR)

The all ESBL producing clinical isolates were analyzed by enterobacterial repetitive intergenic consensus PCR (ERIC2-PCR) as previously reported (Lagha et al., 2014). DNA was amplified using the only one primer ERIC-2: 5'AAGTAAGTGACTGGGGTGAGCG3' (Dumarche et al., 2002), as shown in Table 1. Amplified products were visualized on agarose gels 1.5% stained with ethidium bromide. The profiles of tested clinical isolates were considered different when at least one band differed (Khan et al., 2002).

Conjugation experiments

Conjugation experiments were performed as previously described (Sambrook et al., 1989) with *E. coli* C600 Rif R (Rifampicin resistant) as a recipient strain. A donor strain (clinical isolates) and a recipient strain were grown separately in Brain Heart Infusion broth (Oxoid) at 37°C for overnight. Then the transconjugants were selected on Mueller Hinton agar (Oxoid) containing rifampicin (300 µg/L) and cefotaxime (1 µg/L). All transconjugants were subjected to antimicrobial susceptibility testing and double-disk synergy test.

Plasmid analysis

Plasmids DNA from ESBL strains and their transconjugants were extracted by the method of Kado and Liu (Kado and Liu, 1981). Plasmid profiles were analyzed by electrophoresis in agarose gel at 0.7% and the sizes of the plasmids were determined by comparison with that of plasmids-size standards: Rsa (39 kb), TP114 (61 kb), pCFF04 (85 kb), and (180 kb) as previously described (Robin et al., 2005).

Figure 1. Positive result of a double-disk synergy test for *E. coli* Ec6 (Synergism between amoxicillin-clavulanate and cefotaxime, ceftriaxone, ceftazidime, and aztreonam).

RESULTS

During the study period, 255 non-duplicate clinical strains of enterobacteriaceae were identified. Among these strains, 21 (8.23%) were ESBL positive by double synergy test (Figure 1). The prevalence of ESBL production per species among these tested clinical isolates was the following: 6.91% (13/188) *E. coli*, 10.34% (6/58) *E. cloacae*, 22.22% (2/9) *C. freundii* (Table 2).

The clinical and genetic characteristics, including isolation date, specimen and ward distribution of the 21 ESBL producing isolates are shown in Table 3.

These ESBL-producing strains were isolated from 21 individual patients, consisting of 10 men (47.61%) and 11 women (52.38%) with a mean age of 45.42 years. The patients have been hospitalized during periods between 9

Table 2. Distribution of extended-spectrum β-lactamase producing strains isolated in Laghouat hospital by species and years.

ESBL isolate	2010		2011		2012	
	%	n	%	n	%	n
Escherichia coli	4	3/69	7	4/54	9	6/65
Enterobacter cloacae	0	0/16	9	2/22	20	4/20
Citrobacter freundii	0	0	0	0/5	50	2/4
Total	3.53	3/85	7.4	6/81	13.48	12/89

Table 3. Clinical and genetic characteristics of extended-spectrum β-lactamase-producing *E. coli*, *E. cloacae* and *C. freundii.*

Isolate	Code	Period of isolation	Wards	Sample origin	Sex	Age (years)	β-lactamase pI	β-lactamase gene	Conjugaison
E. coli (n = 13)	Ec1	24/01/2010	Intensive care unit	Catheter	M	53	5.4 + 8.6	CTX-M15	+
	Ec2	18/04/2010	Women medicine	Urine	F	34	7.5 + 8.6	CTX-M15	+
	Ec3	02/05/2010	Intensive care unit	Catheter	F	54	7.5 + 8.6	CTX-M15	-
	Ec4	20/02/2011	Men medicine	Urine	M	46	8.6	CTX-M15	+
	Ec5	06/03/2011	Orthopedics	Urine	M	51	7.5 + 8.6	CTX-M15	-
	Ec6	10/04/2011	Women medicine	Urine	F	32	7.5 + 8.2 + 8.6	CTX-M15, SHV-12	-
	Ec7	07/08/2011	Orthopedics	Pus	M	37	5.4 + 8.6	CTX-M15	+
	Ec8	08/04/2012	Obstetrics-Gynecology	Rectal	F	49	7.5 + 8.6	CTX-M15	-
	Ec9	22/04/2012	Women medicine	Urine	F	39	7.5 + 8.6	CTX-M15	-
	Ec10	24/06/2012	Orthopedics	Urine	M	58	8.6	CTX-M15	-
	Ec11	12/08/2012	Intensive care unit	Catheter	M	56	5.6 + 8.6	CTX-M15, TEM-4	-
	Ec12	02/09/2012	Orthopedics	Urine	F	52	5.6 + 7.5 + 8.6	CTX-M15, TEM-4	+
	Ec13	30/09/2012	Obstetrics-Gynecology	Urine	F	28	7.5 + 8.6	CTX-M15	+
E. cloacae (n = 6)	En1	06/11/2011	Orthopedics	Urine	F	57	5.4 + 7.5 + 8.6	CTX-M15	+
	En2	25/12/2011	Intensive care unit	Blood	F	42	5.4 + 8.6	CTX-M15	+
	En3	08/01/2012	Intensive care unit	Pus	M	55	5.4 + 7.5 + 8.6	CTX-M15	-
	En4	19/08/2012	Orthopedics	Catheter	M	52	5.4 + 7.5 + 8.6	CTX-M15	-
	En5	25/11/2012	Pediatrics	Rectal	F	8 mois	5.4 + 7.5 + 8.6	CTX-M15	+
	En6	09/12/2012	Pulmonology	Urine	M	56	8.6	CTX-M15	+
C. freundii (n = 2)	Cf1	22/01/2012	Women medicine	Urine	F	48	5.4 + 7.5 + 8.6	CTX-M15	-
	Cf2	12/02/2012	Orthopedics	Blood	M	54	5.6	TEM-4	-

and 123 days.

The principal source of isolation was urine (52.38%); following of the catheters source (19.04%), then pus, blood and rectal sources with a percentage of isolation 9.52% for each one.

However, 33.33% of the patients were

Table 4. Antibiotic susceptibility of ESBL-producing clinical isolates (n=21).

Antibiotic	Susceptibility of ESBL-producing isolates: n (%).			Total
	E. coli (n = 13)	*E. cloacae* (n = 6)	*C. freundii* (n = 2)	
Amoxicillin	0 (0)	0 (0)	0 (0)	0 (0)
Ticarcillin	0 (0)	0 (0)	0 (0)	0 (0)
Piperacillin	0 (0)	0 (0)	0 (0)	0 (0)
Cephalotin	0 (0)	0 (0)	0 (0)	0 (0)
Amoxicillin/clavulanic acid	3 (23.07)	1 (16.67)	0 (0)	4 (19.05)
Ticarcilline/clavulanic acid	2 (15.38)	1 (16.67)	0 (0)	3 (14.28)
Cefotaxime	1 (7.69)	1 (16.67)	0 (0)	2 (9.52)
Ceftazidime	1 (7.69)	1 (16.67)	0 (0)	2 (9.52)
Aztreonam	0 (0)	1 (16.67)	0 (0)	1 (4.76)
Cefepime	7 (53.84)	3 (50)	1 (50)	11 (52.38)
Cefpirome	0 (0)	0 (0)	0 (0)	0 (0)
Cefuroxime	0 (0)	1 (16.67)	0 (0)	1 (4.76)
Cefixime	2 (15.38)	1 (16.67)	(0)	3 (14.28)
Piperacillin + tazobactam	11 (84.61)	6 (100)	2 (100)	19 (90.48)
Ceftazidime/clavulanic acid	9 (69.23)	6 (100)	0 (0)	15 (71.42)
Cefoxitin	8 (61.54)	1 (16.67)	0 (0)	9 (42.86)
Imipenem	13 (100)	6 (100)	2 (100)	21 (100)
Kanamycin	8 (61.54)	2 (33.33)	0 (0)	10 (47.62)
Tobramycin	8 (61.54)	2 (33.33)	0 (0)	10 (47.62)
Amikacin	13 (100)	6 (100)	2 (100)	21 (100)
Gentamicin	9 (69.23)	2 (33.33)	0 (0)	11 (52.38)
Netilmicin	9 (69.23)	2 (33.3)	0 (0)	11 (52.38)
Nalidixic acid	6 (46.15)	3 (50)	1 (50)	10 (47.62)
Ofloxacin	4 (30.76)	3 (50)	2 (100)	9 (42.86)
Ciprofloxacin	7 (53.84)	3 (50)	2 (100)	12 (57.14)
Chloramphenicol	3 (23.07)	1 (16.67)	0 (0)	4 (19.05)
Tetracycline	4 (30.76)	3 (50)	1 (50)	8 (38.09)
Colistin	13 (100)	6 (100)	2 (100)	21 (100)
Trimethoprim	6 (46.15)	1 (16.67)	1 (50)	8 (38.09)
Trimethoprim/sulfamethoxazole	6 (46.15)	1 (16.67)	1 (50)	8 (38.09)
Fosfomycin	13 (100)	6 (100)	2 (100)	21 (100)

hospitalized at the orthopedics ward, followed by an intensive care unit (23.8%) and the ward of women medicine (19.04%). In opposite to other hospital wards (General Surgery, Men Medicine, Pulmonology, Obstetrics-Gynecology, Pediatrics), where the prevalence of ESBL-producing strains is low, including one strain was isolated by ward (4.76%).

Antibiotic susceptibilities of ESBL-producing strains showed that all isolates were resistant to amoxicillin, ticarcillin, piperacillin, cephalotin, and cefpirome. Thus high levels of resistance were founds to cefotaxime (90.48%), cefuroxime (95.24%), cefixime 85.72%), ceftazidime (90.48%), aztreonam (95.24%), amoxicillin-clavulanic acid (80.95%) and ticarcilline-clavulanic acid (85.72%).

All ESBL-producing isolates were also multidrug-resistant to others antibiotics and most of them were resistant to: Chloramphenicols (80.95%), trimethoprim (61.91%), and sulfonamides (66.67%). It should be noted that almost half of the isolates were resistant to aminoglycosides (47.62% to gentamicin, 52.08% to tobramycin and kanamycin), and fluoroquinolones (57.14% ofloxacin and 42.86% ciprofloxacin).

Moreover, it is worth noting that all 21 strains (100%) were susceptible to imipenem, amikacin, colistin and fosfomycin. Results of antimicrobial susceptibility testing are shown in Table 4.

In this study, all 21 ESBL-producing isolates were analyzed for their β-lactamases content by isoelectric focusing. It showed the presence of one to five β-lactamase bands with different pIs. The first band of pI5.4 corresponded to the TEM-1-type chromosomal penicillinase. The following bands were of pI: 7.5 (compatible with the OXA-1-type oxacillinase); and pI:

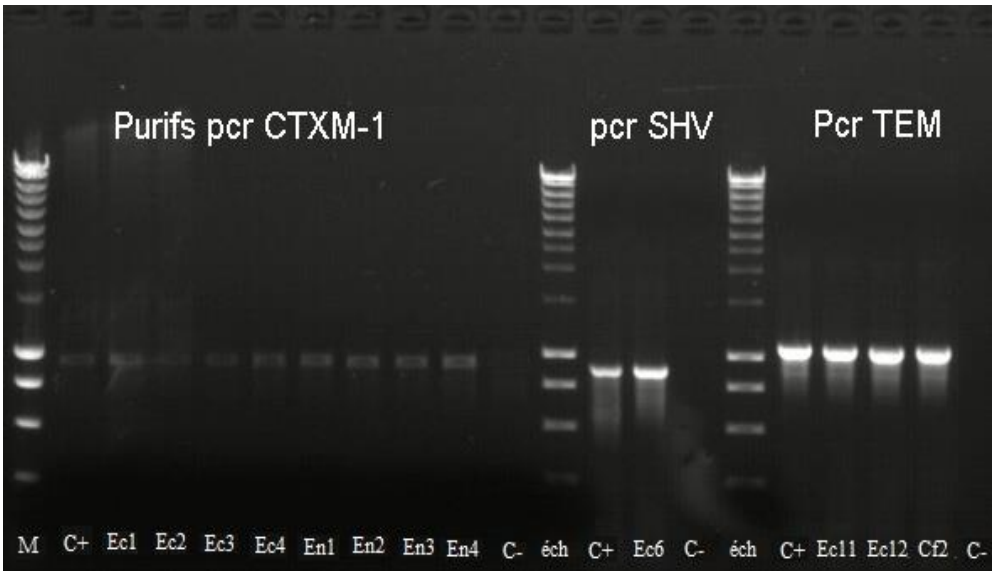

Figure 2. PCR amplification products of *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CTX-M}$ genes (M: molecular weight marker; C+: positive control; C-: negative water control; Ec: *E. coli*; En: *E. cloacae*; Cf: *C. freundii*).

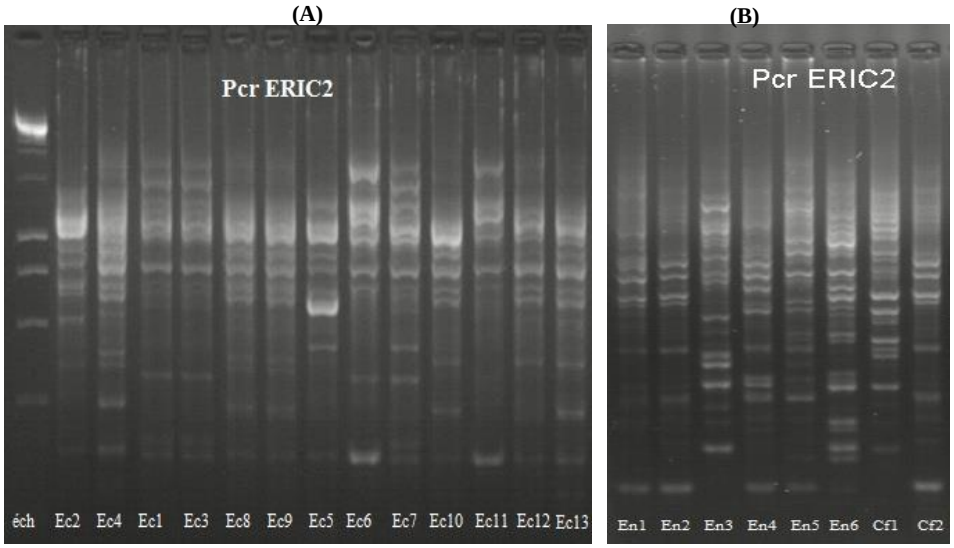

Figure 3. ERIC-PCR profiles of the extended-spectrum β-lactamase-producing isolates obtained with the primer ERIC-2. **(A)** *E. coli* (Ec); **(B)** *E. cloacae* (En) and *C. freundii* (Cf).

5.6, pI: 8.2 and pI: 8.6 corresponded to the different ESBL-types detected.

The genotypic analysis by PCR showed the presence of *bla*$_{SHV}$, *bla*$_{TEM}$, and *bla*$_{CTX-M-1}$ group on the chromosomal DNA of strains (Figure 2). Nucleotide sequence analysis of the *bla*$_{CTX-M}$ genes showed the presence of CTX-M-15 in the twenty ESBL-producing isolates (95.24%). Whereas TEM-4 was detected in three isolates (14.28%) mainly in *E. coli and C. freundii,* and SHV-12 was identified in only one strain (04.76%) of *E. coli* isolated from the women medicine ward. The distribution of the different ESBL types for each strain is shown in Table 3.

ERIC-PCR analysis of genomic DNA from the 21 ESBL-producing clinical isolates revealed that there were 11 different patterns among the 13 *E. coli* isolates (Figure 3A), whereas all strains of *E. cloacae* and *C. freundii* have been different (8 distinct ERIC-PCR patterns were seen in the 8 isolates) (Figure 3B). This indicated clearly heterogeneity in isolates genetic profiles.

Concerning the conjugation assays, 10 ESBL-producing transconjugants were obtained from the 21 ESBL isolates selected, which consisted of six *E. coli* and four *E. cloacae.* All transconjugants also expressed resistance to aminoglycosides (gentamicin and tobramycin), fluoroquinolones, sulfamid and trimethoprim-sulfamethoxazole.

After gel electrophoresis, comparison of the plasmidic content of isolates *E. coli* and *E. cloacae* and their transconjugants showed the presence for each strain, one to four bands, different sizes, between 5 and 180 kb. A common band was observed in all extracts which isolates a high molecular weight > 85 kb (≈130 kb); it indicated that the $bla_{\text{CTX-M-15}}$ gene is carried by this plasmid and transferred between strains.

DISCUSSION

Extended-spectrum β-lactamases producing Enterobacteriaceae (ESBL-E) are emerging worldwide in hospitals and in the community (Bradford, 2001). Their incidence varies according to countries, regions or even hospitals (Arnaud et al., 2015).

In our study, ESBLs were found in 21 (8.23%) of 255 clinical isolates with *E. coli* being the major ESBLs produce (13/21), following of *E. cloacae* (6/21) and *C. freundii* (2/21). This rate of ESBLs isolates is in accordance with those reported in Kingdom of Saudi Arabia (8.9%) (El-Khizzi and Bakheshwain, 2006), but even higher than the rate observed in recent European studies, from 2.4% to 5.1% in France (Toubiana et al., 2016), and 2.9% in Sweden (Kaarme et al., 2013).
Studies conducted in Ethiopia (Mulisa et al., 2016) reported the prevalence rate of 25% of ESBLs producers respectively among Enterobacteriaceae family. These prevalence rates are high as compared to our study which may be attributed to variation in drug management policies or follow other control programs.

Additionally, Latin America, the Middle East, Europe, and the South Pacific displayed a prevalence of ESBL of approximately 10 to 35% (Morrissey et al., 2013; Fernandez-Reyes et al., 2014). However, more than 40% of clinical isolates from Asia were ESBL producers in 2011 (Lukac et al., 2015).

At Laghouat hospital, the patients were hospitalized more frequently in orthopedics wards (33.33%), intensive care unit (23.8%) and the women medicine wards (19.04%). As they were previously-shown, that orthopedic surgical site infections are often associated with substantial morbidity and exorbitant costs, and are challenging to treat, especially in case of multi-resistant pathogens or presence of implants (Martinez-Pastor et al., 2010). Additionally, many studies in Intensive care units have established risk factors for the acquisition of infection due to ESBL producing *E. coli* and even to other bacterial species (Oteo et al., 2013).

Moreover, it is worth noting that, the principal source of isolation the ESBL-producing isolates in our study was urine with the proportion 52.38%; our results are in accordance with other recent studies realized on a group of 124 Enterobacteriaceae isolates resistant to third generation cephalosporin, and collected in distinct health care facilities of different Portuguese regions, which have even described a 58.9% clinical strains were isolated from urine (Jones-Dias et al., 2014). However, studies have shown that ESBL producing uropathogens have their reservoir in the digestive tract (Anil Kumar and Babu, 2013).

The major risk factors found in this study were length of hospitalization, the hospital ward where the ESBL-strains was isolated (orthopedics, intensive care unit and the women medicine wards), urinary tract, and antibiotic therapy, as previously reported (Dayan et al., 2013)

Our antimicrobial susceptibility analysis of the all ESBL-producing isolates found highly prevalent resistances against to the majority of β-lactams, and even to others tested antibiotics: Gentamicin, tobramycin, ofloxacin, chloramphenicols, trimethoprim, and sulfonamides, which confirmed the presence of multidrug-resistant isolates in this hospital. Unfortunately, comparable results of the susceptibility rate to these molecules were also reported by Rakotonirina et al. (2013).

This correlates with other studies, where many ESBL producers are multi-resistant to non-β-lactam antibiotics, including fluoroquinolones and aminoglycosides (Livermore et al., 2007), trimethoprim, tetracyclines, sulfonamides, and chloramphenicol, which are often encoded by the same plasmids that determine the ESBL (Karisik et al., 2006).

Consequently, effective antibiotic therapy for treating these infections is limited to a small number of drugs, such as carbapenems and thus increasing the chance of resistance to carbapenems among the Enterobacteriaceae (Pitout, 2010).

In this study, among the twenty one ESBL-producing clinical strains isolated, $bla_{\text{CTX-M-15}}$ was the most commonly detected genotype in all clinical isolates (95.24%).

The blaCTX-M-15 ESBL gene is considered to be the most prevalent ESBL worldwide, as it is found in a Tunisian study analyzed 32 ESBL *E. coli* isolates collected during a 10-month period, which reported the emergence of CTX-M-15 in 97% (31/32) of isolates; and 81% (26/32) also harbored TEM-1 (Réjiba et al., 2011).

According to the previously studies realized, among ESBL-producing Enterobacteriaceae in Algeria County, CTX-M-15 with CTX-M-3 enzymes were most frequently reported in the west (Baba Ahmed-KaziTani et al., 2013), centre (Ramdani-Bouguessa et al., 2006) and east of Algeria (Gharout-Sait et al., 2012). The rapid dissemination of CTX-M-15 producing Enterobacteriaceae were reported in a number of countries is a significant public health concern (Bonnet,

2004; Livermore et al., 2007).

Thus, in our study, 14.28% of the clinical isolates harbored *bla*$_{TEM-4}$, followed *bla*$_{SHV-12}$ which is also distributed, but only in a single strain of *E. coli* with rate of 4.76%. This SHV-12 type extended-spectrum β-lactamase is most often found in Asia (Kim et al., 1998) and including Africa (Kasap et al., 2010). It is already found in Algeria, as shown previously in *E. cloacae* (Iabadene et al., 2008), and *K. pneumonia* (Berrazeg et al., 2013).

However, the spread of TEM-4 enzyme is very rare in Algeria; their presence is reported only in one study by Kermas et al. (2012) where this enzyme is detected in *Salmonella enterica*.

The molecular typing by ERIC-PCR revealed that the majority of ESBL-producing isolates from our hospital showed distinct genetic profiles, with the presence of 19 different patterns among the 21 genetic profiles. These isolates were considered genetically unrelated. This property explains the easy horizontal dissemination of *bla*$_{CTX-M-15}$ -harboring plasmids, a high molecular weight > 85 kb (≈130 kb); and their emergence between different strains.

This result correlates with previous studies in Algeria, where showed that *bla*$_{CTX-M-15}$, was carried by conjugative plasmid of high molecular weight, vary from 85 kb (Messai et al., 2008), to ≥ 125 kb (Nedjai et al., 2012). Already, Messai et al. (2008) showed that the CTX-M-15 enzyme was developed from CTX-M-3 under Algerian clinical context.

This dissemination of the CTX-M-15-type ESBLs is not restricted to the nosocomial setting but also involves the community. This phenomenon is acting to modify the epidemiology of ESBLs, whereas those enzymes were, previously, mostly restricted to the nosocomial setting (Rossolini et al., 2008).

This study is the first report conducted on ESBL-producing strains of *E. coli*, *E. cloacae* and *C. freundii*, isolated from various clinical specimens in Laghouat hospital, Southern Algeria; that demonstrates such a high prevalence of CTX-M-15 enzymes among clinical strains. Although, TEM-4 and SHV-12 are the less frequent enzymes isolated in some strains of *E. coli* and *C. freundii*. All this indicates the dissemination of multidrug resistant isolates in Laghouat, in different hospital wards and probably in the community.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Performance of dried blood spot (DBS) PUNCHER and dried blood spots to measure HIV-1 viral load

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The use of plasma is an obstacle to realize HIV-1 viral load in sub-Saharan Africa. In this context, the dried blood spot (DBS) is an interesting tool for sample collections. This approach was tested using a DBS hole-punch device (PUNCHER). Plasma and DBS samples were obtained from 102 patients, comprising 17 HIV-1 negative patients and 85 HIV-1 infected patients. The PUNCHER's performance used to cut DBS was evaluated with the following criteria: ease of use, time savings and safety. VL was measured in parallel on plasma and DBS samples using NucliSENS EasyQ HIV-1. The correlation between plasma and DBS results was strong (R = 0.91; P < 0.001). The mean difference (± standard deviation) was -0.59 ± 0.52 $\log_{10}$ copies/ml. The sensitivity and specificity of DBS were 91.3% (n= 74) for the 81 VL detectable samples and 100% for the 21 VL undetectable samples, respectively. On a scale of 10, the PUNCHER's performance scored 9.3 for ease of use, 8.6 for time savings and 10 for safety. PUNCHER is highly efficient at cutting DBS, and the VL resulting from DBS correlated well with those obtained from plasma.

Key words: Puncher, dried blood spot (DBS), viral load, performance.

INTRODUCTION

In developed countries, viral load (VL) is an essential assay for monitoring the human immunodeficiency virus type 1 (HIV-1) infection, especially for evaluating the efficacy of antiretroviral treatment (ART) (Mellors et al., 1997; Yilmaz, 2001). In sub-Saharan Africa, the use of VL monitoring to detect treatment failure is the major challenge to improve HIV management. Access to this assay faces several obstacles: cost of equipment and reagents, availability and stability of energy. If VL measuring equipment exists in these countries, it is more often only available in reference laboratories far from peripheral sites, posing the problem of accessibility to people living with HIV. Then, the use of dried blood spot (DBS) represents an alternative for the collection and transport of samples as compared to plasma, which requires more restrictive conditions, transport without delay and storage at -80°C (Cassol et al., 1997; Brambilla et al., 2003; Alvarez-Muñoz et al., 2005; Kane et al, 2008 ; Johannessen, et al 2009; Arredondo et al, 2012). In most laboratories, DBS are cut with a pair of scissors,

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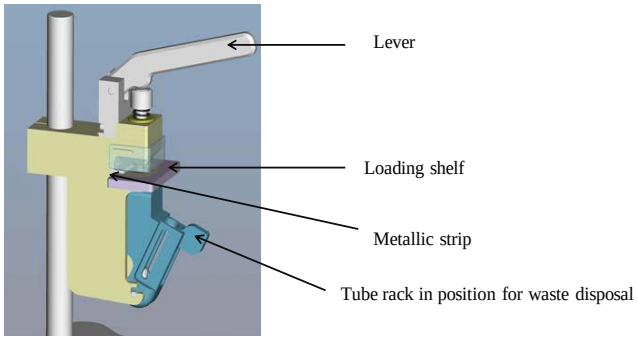

Figure 1. Puncher –bioMérieux.

with a risk of injury while cutting or decontaminating the scissors. In addition, discomfort from the long use of scissors limits the number of DBS that are cut.

The objectives of this study were, to evaluate the performance of the puncher, a new method of cutting DBS (weight = 4.6 kg; length = 48.5 cm; width = 21 cm; Figure 1; http://www.biomerieux-diagnostics.com) according to 7 criteria and to investigate the performance of DBS in HIV-1 RNA quantification against the standard plasma viral load assay.

METHODS

A total of 102 patients, comprising 17 HIV-1 negative patients and 85 infected patients (58% of whom were antiretroviral therapy naive), were recruited at Sylvanus Olympio University Hospital in Lomé (Togo) over a period of six months, January to June 2013. After obtaining written informed consent, five milliliters of whole blood was drawn from each patient by venipuncture and collected in tubes with EDTA. DBS were prepared by dispensing 50 µl of blood per spot (5 spots per card) onto filter paper cards (Whatman no. 903; Schleicher and Schuell, BioScience GmbbH, Barcelona, Spain). The spotted filter papers were allowed to dry at room temperature for 4 to 6 h in a hood. The DBS were stored in zip-lock plastic bags with a silica gel desiccant at room temperature for 15 days before further processing and assaying. The remaining blood sample was centrifuged at 1500 xg and plasma was stored at -80°C until testing.

The DBS were cut with the PUNCHER according to the manufacturer's instructions. During the use of the PUNCHER, the following performance-related parameters were evaluated: speed of handling, number of manipulations to cut the spots, speed of positioning the card; risk of contamination associated with handling. To avoid contamination of one sample by another, 3 white spots of the same blotting paper were cut after punching 2 spots of a DBS sample before moving on to the next sample. Seventeen (17) HIV-1 negative samples were included in the study to evaluate the contamination risk by using the PUNCHER. Criteria for PUNCHER evaluations were defined taking into account the difficulties associated with using scissors to cut DBS. The use of the PUNCHER was carried out by one lab technician. For each parameter, a score between 1 and 10 was awarded after each sample: 10 meaning completely in line with expectations.

HIV-1 RNA isolation from DBS and plasma was performed using 100 µl (2 spots) and 500 µl of samples, respectively. HIV-1 RNA was extracted from the same patient in plasma and DBS according to the NucliSENS miniMAG procedure. VL was measured by NucliSENS HIV-1 EasyQ version 2.0 (bioMérieux, Lyon, France). All RNA values are reported as $\log_{10}$-transformed copy numbers of HIV RNA per ml of DBS or plasma. Viral load was stratified into three levels (undetectable VL, VL < 5000 copies/ml and VL > 5000 copies/ml). Sensitivity and specificity of DBS viral load using plasma assay as the gold standard was assessed at all three viral load strata. Pearson correlation analysis was performed, as well as Bland-Altman analysis to examine the level of agreement between the two tests (Bland et al., 1986). Bland-Altman analysis was performed using MedCalc version 9.5.0.0 (MedCalc Software, Mariakerke, Belgium). With the given sample size that was used for Bland Altman analysis, the 95% CI for the limits of agreement were ± 0.11 $\log_{10}$ copies/ml. This narrow range in the precision of the limits of agreement was deemed to be clinically acceptable. Differences were considered significant only when P values were <0.05. The Ministry of Health of Togo (No. 0411/2012/MS/CAB/ DGS/DPLET/CBRS) and National ethic committee of Togo (Comité de bioéthique pour la recherche en santé, No. 001/2012/CBRS) approved the study.

RESULTS

The plasma results showed that all HIV-1 negative samples (n=17) were undetectable for VL results. From 85 infected patients, 81 (95.3%) were detectable VL (Table 1), including 24 VL < 5000 copies/ml and 57 VL > 5000 copies/ml. The correlation between DBS and plasma was high (R = 0.91; P < 0.001) (Figure 2). The mean difference and standard deviation in samples with a VL < 4 $\log_{10}$ (n = 30) was 0.65 (0.40); 0.60 (0.25) for samples between 4-5 $\log_{10}$ (n = 26) and 0.59 (0.15) for samples with a VL > 5 $\log_{10}$ (n = Bland-Altman plot illustrates the agreement between plasma and DBS (Figure 3). The mean difference for quantitative 25). Overall, 49 pairs of plasma and DBS (49%) had a difference of more than 0.5 log copy, and for 14 pairs of samples (14%), the difference was greater than 1 $\log_{10}$ copy. The corresponding data between measurements (DBS minus plasma) was -0.59 $\log_{10}$ copy (standard deviation, 0.52 $\log_{10}$ copies/ml). The sensitivity of DBS was 91.3% (n= 74) for the 81 VL detectable samples. Ten results from DBS were < 5000 copies/ml among patients who had VL > 5000 copies with plasma; for 6 of these 10 samples, the VL from plasma was between 5000 and 7000 copies/ml and for 4 samples the VL was > 11000 copies/ml. The specificity of DBS versus plasma was 100% for the 21 VL undetectable samples (17 HIV-1 negative samples and 4 HIV-1 positive samples).

On a scale of 10, PUNCHER scored 9.3 for ease of use, 8.6 for time savings (Table 2). Concerning the rapidity of cutting, 124 DBS corresponding to 62 patients were cut in one hour using the puncher. As compared to archives data (unpublished data), 88 DBS corresponding to 44 patients were cut using scissors in the laboratory. No contamination was observed, all 17 negative samples were found negative although randomly tested between samples with high viral loads. The absence of contamination proved the efficacy of the decontaminating protocol.

Table 1. Summary of the HIV-1 viral load results of 102 samples from plasma and DBS.

		Plasma			Total
		Undetectable	VL* < 100 copies	Quantifiable	
DBS	Undetectable	19	3	3	25
	VL* <100 copies	0	0	1	1
	Quantifiable	2	1	73	76
Total		21	4	77	102

*Viral load.

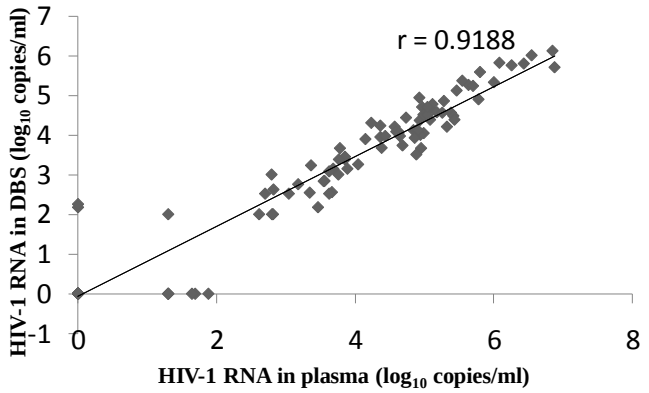

Figure 2. Linear regression comparing HIV type 1 RNA levels obtained by testing 102 paired plasma and DBS

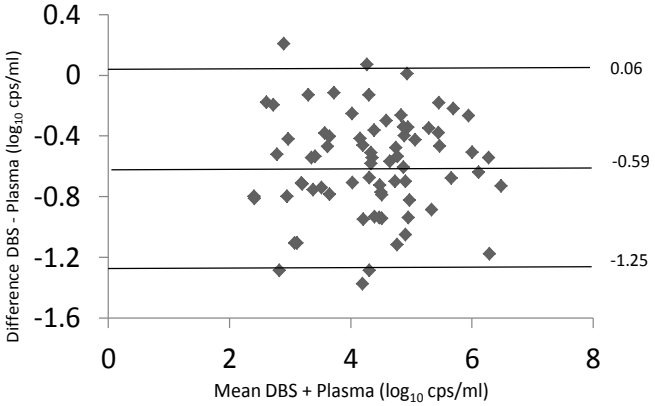

Figure 3. Bland and Altman analysis of viral load values comparing DBS versus plasma (n = 73) using NucliSENS EasyQ HIV-1 assay.

DISCUSSION

The use of DBS specimen as source for diagnostic test has become increasingly popular in recent years. DBS has been used to identify genetic and metabolic disorders in neonates, detection of HIV-1 antibody, and HIV-1 DNA for infant diagnosis of HIV infection. The WHO recommends the use of DBS for HIV drug resistance surveillance for monitoring transmitted drug resistance in resources limited settings (WHO, 2010).

In this study, a new tool to cut DBS replacement scissors wa evaluated in order to validate its use in routine practice. As compared to scissors, the performance of the puncher concerning ease of use, time savings and rapidity of cutting was better. But the data obtained for the scissors are archive data.

The VL results were divided into two groups based on the new WHO recommendations; patients on high active antiretroviral therapy for at least 4 weeks and with a VL > 5000 copies/ml are considered to be in treatment failure (WHO, 2012). The results showed a strong correlation between plasma and DBS. But the small sample size included in this study may limit the accuracy of the results; however, significant correlation and limits of agreement of two assay methods found in this study reinforce the usefulness and feasibility of utilizing DBS as method for clinical viral load monitoring of patients on ART or HIV-1 early diagnosis.

Previous data report correlations range from 78 to 99% between DBS and plasma according to different analyzers for the measurement of VL, and sometimes with different extraction methods (Brambilla et al., 2003; Johannessen et al., 2009; Garrido et al., 2009; Marconi et al., 2009; Mbida et al., 2009; Hamers et al., 2009; Bertagnolio et al., 2010; Johannessen et al., 2011b; Neogi et al., 2012). In this study, DBS were stored at room temperature (28 to 35°C) for 15 days before being handled, and were never frozen. 15 days was set as storage time at room temperature for DBS in this method to be used by the HIV care centers; which gives a time to transport DBS to the national reference laboratory.

HIV-1 RNA quantification from DBS has shown good stability under different temperature and storage conditions ranging from ambient to -70°C (Cassol et al., 1997; Brambilla et al., 2003; Alvarez-Muñoz et al., 2005; Kane et al., 2008; Marconi et al., 2009; Monleau et al., 2010). Nevertheless, subsequent studies should define the DBS VL values at which the patient will be considered either in remission or in virological failure.

In conclusion, the performance of the PUNCHER is excellent. It can therefore be recommended tolaboratories

Table 2. Performance of the DBS PUNCHER.

Parameter	Total no. of points for 102 DBS punches*	Score out of 10
Ease of use		
Handling	966	9.6
Number of manipulations to punch out the spots	890	8.9
Time savings		
Speed of handling	909	9.1
Speed of positioning the card	829	8.2
Manipulation time (card to lysis tube)	867	8.7
Safety		
Risk of contamination associated with handling	1000	10
Method of decontamination	908	9

*Each DBS Punched was scored between 1 and 10; 1 means does not meet expectations and 10 means completely in line with expectations.

handling DBS to avoid the disadvantages and risks related to the use of a pair of scissors. The results is recommended also, because of its good performance for measuring viral load in tropical climatic conditions. DBS can be used as an alternative sampling method for viral load monitoring in resource-limited settings. Further studies of operational research to apply these findings within a clinical setting on a large scale will be useful.

Conflict of interest

The authors have not declared any conflict of interest.

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Full Length Research Paper

Prevalence and antibiotic susceptibility of *Staphylococcus aureus* from lactating cow's milk in Bahir Dar dairy farms

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Staphylococcus aureus **is competitive in milk and dairy environments; pathogenic strains have been found to cause disease in their host throughout the world. Therefore, this study was designed to assess the prevalence of *Staphylococcus aureus* and determine their antibiotic susceptibility from lactating cow milk. A cross-sectional study was conducted in Bahir Dar dairy farms from October 2012 to March 2013. A total of 218 raw milk samples from lactating cows were collected from dairy farms in Bahir Dar, North-West Ethiopia. The *S. aureus* bacteria were isolated on Mannitol Salt agar (Becton, Dickinson) where yellow colonies were selected and counted and then maintained for antibiotic susceptibility tests. Susceptibilities of the isolates were tested against 9 antibiotics using the Kirby-Bauer disc diffusion method. Overall, 98 milk samples (45%) were found to be contaminated with S. aureus with average count varying between 3.3×10^2 to 7.2×10^4 CFU/ mL. *S. aureus* prevalence showed significant variation among cows of different hygienic conditions ($p < 0.05$). *S. aureus* isolates were highly susceptible to ciprofloxacin (100%) followed by gentamycin (96%), chloramphenicol (74%), erythromycin (68%), trimethoprimsulfamethoxazole (66%) and tetracycline (60%). In contrast, isolates were highly resistant to penicillin (94%) and cephoxitin (62%). Most of the isolates (96%) were resistant to one or more antibiotics. In general, the results of the present study revealed that milk provided to the consumers in the city was found to be less hygienic. Thus, farmers should ensure strict personal hygiene and that of animals, and general sanitary condition of the farms should be improved and maintained.**

Key words: Antibiotics, Bahir Dar, milk, *Staphylococcus aureus*, susceptibility.

INTRODUCTION

Most foods contain viable bacteria unless thoroughly heated or pasteurized. Otherwise, food serves as an important vehicle for transmission of pathogenic organisms to consumers. Contamination of food products with pathogenic organisms may influence considerably their harmlessness, endanger the health of consumers

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and decrease shelf life, resulting in food borne infections, intoxications and economic losses from food spoilage (HPSC, 2012).

Milk is one of the most important foods for human beings and also universally recognized as a complete diet due to its essential components (Javaid et al., 2009). However, health risk to consumers can be associated with milk, due to the presence of zoonotic pathogens and antimicrobial drug residues (Vyletělova et al., 2011). Milk is an excellent bacteriological medium for a large number of microorganisms, including *Staphylococcus aureus*. When milk is drawn from the udder of a healthy animal, it contains organisms that have entered the teat canal through its opening. *S. aureus* bacteria are mechanically flushed out during milking. The number ranges during milking between several hundred to several thousand per milliliter (Farzana et al., 2004).

Milk can be contaminated by *S. aureus* when there is infection of the mammary gland or by bad hygiene habits, such as coughing or sneezing and not washing hands when handling milk storage equipment, during or after milking, and in some cases, human activity is responsible for the contamination, as this bacteria colonizes the nasal pathways in human beings (Fagundes et al., 2010). The micro biota found on the hands and on the uniform of food handlers, especially of milk, is a reflection of hygiene habits as the single most important factor in the contamination of milk (Lingathurai and Vellathurai, 2011). *S. aureus* is competitive in milk and dairy environment. Pathogenic strains are usually coagulase-positive and have been found to cause disease in their hosts throughout the world. The presence of *S. aureus* shows up unsanitary conditions in the cattle herd and counts above 10^3 CFU/mL in milk increase the risk of staphylococcal toxin production more resistant to the heat processes of pasteurization (Tortora et al., 2005). Diseases in cattle caused by *S. aureus* are ranging from simple abscesses and mastitis to the more severe toxic shock syndrome (Tesfaye et al., 2010).

The growth of *S. aureus* and potential production of heat-stable enterotoxins with respect to the food matrices and conditions of food preparation represent a potential, even actual threat of a public health problem residing in food poisoning outbreaks. That is why the control of *S. aureus* growth during the fermentation of young raw milk cheese means prevention against staphylococcal enterotoxin production is recommended (Charlier et al., 2009).

S. aureus in raw milk comes from cows with mastitis, from handlers or from deficient hygiene (Fagundes et al., 2010). When found in milk, high levels of contamination can be reached quickly under favorable conditions. Its presence in foods can be a risk to human health, causing a public health problem, as these bacteria produces toxins that can cause toxic food infections (Quintana and Carneiro, 2006). The capacity to coagulate plasma, the principal characteristic of the *S. aureus*, is highly correlated to the capacity to produce enterotoxins harmful to the tissues of the contaminated host (Murray et al., 2006).

S. aureus mastitis is a serious problem in dairy production and infected animals may contaminate bulk milk. *S. aureus* is still an important cause of food borne intoxications worldwide (Ertas et al., 2010). The ability of *S. aureus* to grow and produce staphylococcal enterotoxins (SEs) under a wide range of conditions is evident from the variety of foods implicated in staphylococcal food poisoning (SFP) (Le Loir et al., 2003). SFP is suspected when the symptoms including nausea, violent vomiting, abdominal cramps and diarrhea affect the patients between 1 and 8 h after food consumption (Balaban and Rasooly, 2001). In addition, the prevalence of mastitis and its associated pathogens in animals can be reduced by improving on the farm management techniques within the dairy industry (Pitkälä et al., 2004). It is thus of paramount importance to ensure that proper hygienic practices are enforced in both the area where the animals are kept and the milking environment (Lingathurai and Vellathurai, 2011).

Although it is difficult to control mastitis caused by *S. aureus* with antibiotics only, various antimicrobial agents antifungals are constantly being used to treat this disease in cattle. This practice results in the development of antibiotic-resistant strains (Thaker et al., 2013). The usage of antibiotics correlates with the emergence and maintenance of antibiotic-resistant traits within pathogenic strains (Shitandi and Sternesjö, 2004). These traits are coded for by particular genes that may be carried on by the bacterial chromosome, plasmids and transposons or on gene cassettes that are incorporated into integrons **(**Rychlik et al., 2006), thus are easily transferred among isolates. Multiple antibiotic resistant *S. aureus* strains have been isolated from milk obtained from cattle, beef and human samples in many parts of the world (Pesavento et al., 2007). The prevalence of antibiotic resistance usually varies between isolates from the different sampled stations and even between isolates from different herds on the same farm (Waage et al., 2002). Consequently, the good quality milk is a challenge that can be overcome provided that basic care is taken at the source of production (Alves, 2006). The importance of microorganisms in the milk means that their microbial contamination index can be used to judge the quality, as well as the sanitary conditions of its production and the health of the herd (Guerreiro et al., 2005).

Significance of the study

Antibiotic-resistant *S. aureus* isolates poses a severe challenge to both veterinary and health professions and dairy cattle producers because of their negative impact on therapy. Therefore, determination of levels of *S. aureus* and an evaluation of the antibiotic-resistant

phenotypes of the isolates could serve as a tool for determining the hygiene standards implemented during milking. Data on antibiotic resistance could also be used to characterize these opportunistic pathogens, which may further limit the risks associated with the consumption of contaminated milk and its products. There is few published data about bovine mastitis in dairy farms of Bahir Dar and its surrounding by Bitew et al. (2010) and Almaw et al. (2008). However, there is no report about the current status of the prevalence and susceptibility assay specifically of *S. aureus* from healthy lactating cow's milk in dairy farms of Bahir Dar.

Objectives of the study

General objective

To determine the prevalence and antibiotic susceptibility of *S. aureus* from lactating cow milk in Bahir Dar dairy farms

Specific objectives

1. To determine the prevalence of *S. aureus* in milk of cow in Bahir Dar Dairies
2. To evaluate the antibiotic susceptibility patterns of *S. aureus* isolates
3. To assess some associated risk factors for contamination of cow milk by *S. aureus* and
4. To assess the time course of growth of *S. aureus*

MATERIALS AND METHODS

The study area

The study was conducted in dairy farms of Bahir Dar. Bahir Dar is the capital of Amhara National regional state and is located at about 578 km North-West of Addis Ababa which is the capital of Ethiopia. It has a total population of 256,999 (CSA, 2011). The area of the town is 160 km^2.. Geographically the region is located between 9°20' and 14°20' latitude North and 30°20' and 40°20' longitude East. It has a summer rainfall; the highest rainfall is between June and September and a winter dry season (December to March) with mean annual rainfall of 1200 to 1600 mm, mean temperature 10 to 20°C and an altitude at 1500 to 2300 m above sea level (Bureau of Agriculture, 2006).

Study design

Cross sectional study was conducted. The study was conducted on dairy farms found in Bahir Dar, North-West Ethiopia between October 2012 and March 2013. The study populations were all lactating Holstein crossbred cows. The laboratory study was done in Microbiology Laboratory, Bahir Dar University.

Sample size determination

The desired sample size was calculated according to the formula given by Thrusfield (2005) cited in Tariku et al. (2011). About 218 lactating cows were considered to be as a sample.

Sample collection

Aseptic procedures were followed for milk sample collection, handling, and transportation to the Microbiology laboratory, Bahir Dar University. Samples of approximately 10 ml of fresh milk were collected from dairy farms by using sterilized test tubes and ice box. Two hundred and eighteen (218) observational check list copies were prepared and then observation was implemented to identify potential risk factors and evaluate their effects on the quality of the milk. Data on each farmer's herd has been collected in a properly designed data collection manner.

Isolation of *S. aureus* from milk samples

The samples were processed immediately upon arrival using aseptic techniques. Ten-fold serial dilutions (10^{-1} to 10^{-3}) were performed using sterile saline solution and 1 ml from each dilution was taken aseptically and pour plated in to three plates of mannitol salt agar (Oxoid, England). The plates were incubated aerobically at 37°C for 18 to 24 h (Quinn et al., 2004). After growth of organisms, plates with yellow colonies with bright yellow zones were counted. The mean number of *S. aureus* cfu/ml of milk for each original sample was calculated by taking the average number of bacteria per milliliter from the three dilutions. The number of cfu/ml was then calculated.

A colony of all Staphylococci positive samples was sub-cultured on mannitol salt agar (MSA) and incubated for 24 h at 37°C and the isolates were retained for antibiotic susceptibility test as slants on nutrient agar.

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed by the Kirby-Bauer disc diffusion method. Antibiotic susceptibility tests were performed on 50 *S. aureus* isolates to determine their antibiotic-resistance profiles (Kirby et al., 1966). *S. aureus* from the agar slants were inoculated in tryptose soy broth and incubated at 37°C. Fresh overnight cultures were used for antibiotic sensitivity tests. An aliquot from each isolate suspension was spread plated by sterilized swab on Mueller Hinton agar (Oxoid, England). Antibiotic discs were gently pressed onto the inoculated Mueller Hinton agar (Oxoid, England) to ensure intimate contact with the surface and the plates were incubated aerobically at 37°C for 18 to 24 h (CLSI, 2011). Antimicrobial susceptibility test was conducted using 9 antibiotics. The antibiotics used were erythromycin (15 µg), penicillin G (10 IU), gentamicin (10 µg), trimethoprim-sulfamethoxazole (25 µg), chloramphenicol (30 µg), vancomycin (30 µg), tetracycline (30 µg), cephoxitin (30 µg) and ciprofloxacin (5 µg). The susceptibility of the *S. aureus* isolates (Inhibition zone diameters) to each antibiotic agent were measured and the results were categorized as either susceptible, intermediate or resistant based upon interpretive criteria developed by the Clinical and Laboratory Standards Institute (CLSI) to antimicrobials (CLSI, 2011).

Growth pattern of *S. aureus* bacteria

The selected *S. aureus* isolate was evaluated for growth pattern in nutrient broth. To assess the growth pattern of the *S. aureus* isolate, exponentially growth culture was inoculated into liquid nutrient broth and incubated. Growth was determined

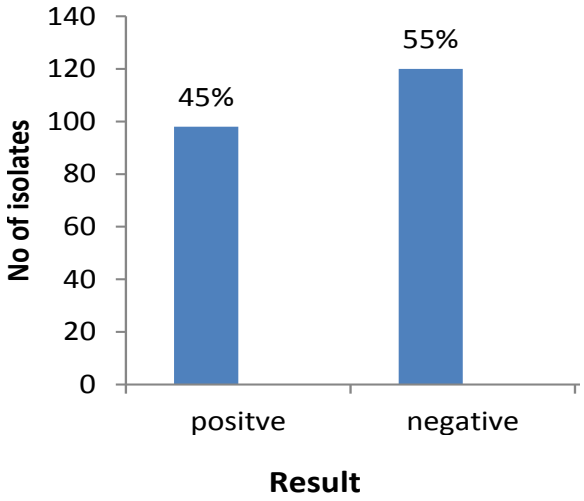

Figure 1. Prevalence of *S. aureus* in Bahir Dar dairy farms (n=218). Positive: *S. aureus* contaminated samples; Negative: Samples free from *S. aureus.*

Table 1. Viable count of *S. aureus* in lactating row cow milk in Bahir Dar Dairy farms.

Number of contaminated samples	Corresponding viable count (CFU/ml)
16	10^2
21	10^3
61	10^4

turbidimetrically after interval of 6 h for three days by measuring optical density (at 540 nm wave length) using spectrophotometer (Appendix Table 1).

Data analysis

The data was analyzed using SPSS 16 software. A *p* value less than 0.05 were considered as statistically significant. Descriptive statistics such as percentages and frequency distributions were used to describe the nature and the characteristics of the data. The association between prevalence of *S. aureus* and the associated risk factors were compared by using Chi square test.

Ethical clearance

This study has been ethically cleared by Bahir Dar University Biology Department. The objectives of this study were well explained to all participating smallholder dairy farmers who all expressed their consent to participating in the study.

RESULTS AND DISCUSSION

Prevalence of *S. aureus*

Out of 218 milk samples (from a total 218 cows) analyzed, 98 of them were found to be contaminated by *S. aureus*, corresponding to 45% of samples and 120 (55%) of them were free from *S. aureus* (Figure 1), with an average viable count varying between 3.3×10^2 to 7.2×10^4 CFU/ mL. A total of 98 potential isolates were sub-cultured and further analyzed for antibiotics susceptibility. The results demonstrated the presence of *S. aureus* and the levels of contamination with *S. aureus* were high in milk samples. The frequency of *Staphylococcal* counts varied between different herds of farmers. Out of the contaminated samples of raw milk, 16 had levels of *S. aureus* corresponding to 10^2 CFU/mL; 21 had levels of 10^3 CFU/mL and 61 samples had count of 10^4 CFU/mL (Table 1).

Counts of *S. aureus* above 10^3 increase the probability of production of staphylococcal toxins that are resistant to boiling carried out in the homes when buying raw milk, and to the pasteurization processes (Tebaldi et al., 2008). Considering this, most of the samples had numbers of *S. aureus* above 10^3 CFU/mL, thus such milk consumed in Bahir Dar has a serious risk to the health of the population.

The presence of *S. aureus* also shows deficient sanitary conditions of the cattle herd. The presence of *S. aureus* in milk results from Bahir Dar dairy farms are similar to those of Oliveira et al. (2011), where out of 50 samples of raw milk in Brazil, 68% samples were contaminated with *S. aureus*. Similarly, Daka et al. (2012) analyzed a total of 160 milk samples and found 78 samples positive for *S. aureus* (49% of total isolates) in

Table 2. Risk factors for the prevalence of *S. aureus* in milk of lactating cows, Bahir Dar dairy farms.

Associated risk factors	Negative No. (%)	Positive No. (%)	Total (%)	χ2 (p-value)
Washing teats and udder before milking				
Yes	117 (53.7)	52 (23.85)	169(77.5)	61.137(0.000)
No	3 (1.4)	46 (21.1)	49(22.5)	
Milkers wash their hands after milking each cow				
Yes	38 (17.43)	6 (2.75)	44 (20.18)	21.85(0.000)
No	82(37.6)	92 (42.2)	174 (79.8)	
Milkers use any agent to clean their hands				
Yes	5 (2.3)	0 (0)	5 (2.3)	4.179 (0.049)
No	115(52.75)	98 (44.95)	213 (97.7)	
Floor hygiene				
Good	37 (17)	2 (0.9)	39(17.9)	30.444(0.000)
Poor	83 (38)	96 (44)	179 (82)	
Hair				
Covered	41 (19)	14(6)	55 (25)	11.303(0.001)
Not covered	79(36.2)	84 (38.5)	163 (74.77)	
Milking utensils cleaned				
Yes	33 (15)	6 (3)	39 (18)	16.783(0.000)
No	87 (40)	92 (42)	179 (82)	

+Total number of cows observed =218, while milking by 41 milkers; Positive: *S. aureus* contaminated samples; Negative:- Samples free from *S. aureus.*

Hawasa, Ethiopia. Moreover, Mekonnen et al. (2011) observed that 39.5% of milk samples in total of 200 milk samples were contaminated with *Staphylococcus* species in Debre Zeit, Ethiopia.

In contrast, our study had very low count of *S. aureus* compared to the previous study conducted by Ateba et al. (2010) in South Africa who reported 100% prevalence of *S. aureus* in the milk samples analyzed. Quintana and Carneiro (2006) analyzed raw milk in Morrinhos, in the state of Goias and found that 28.5% of the samples had *S. aureus* above 10^4 CFU/mL. Similarly, Wani and Bhat (2003) examined 100 milk samples and recovered 95 bacterial isolates. Out of these bacterial isolates 45% were *S. aureus.* These results are similar with the results obtained in this study.

Contamination in milk was also detected in other countries, with similar results. In Palestine, 48 (36.9%) of samples were positive for *S. aureus* out of 130 samples (Farhan and Salk, 2007). In Turkey, Ekici et al. (2004) found 18.18% of samples contaminated of the total samples studied. In the north of Morocco, Bendahon et al. (2008) isolated 40% of *S. aureus* in raw milk from samples.

In India, 61.7% *S. aureus* were detected in 60 samples of raw milk (Lingathurai and Vellathurai, 2011) and D'Amico and Donnelly (2010) found 29% samples positive for *S. aureus* in Vermont, in the United States.

Associated risk factors for the contamination of milk by *S. aureus*

The main source of the infection is the udder of infected cows which is transferred via milker's hands, utensils, towels and the environment in which the cows are kept (Radostitis et al., 1994). *S. aureus* has adapted to survive in the udder and establish chronic and subclinical infections. From there it is shed into the milk, which serves as a source of infection for healthy cows during the milking process. The high prevalence of *S. aureus* can most likely be attributed to the wide distribution of the organism inside mammary glands and on the skin of teats and udders (Radostitis et al., 1994). The mammary gland is more susceptible to new infection during the early and late dry period, which may be due to the absence of udder washing and teat dipping, which in turn may have increased the presence of potential pathogens on the skin of the teat (Radostits et al., 2000). The associated risk factors for the contamination of milk in this study are indicated in Table 2. As Table 2 indicated, the majority of the milkers (97.7%) did not use any kind of agent soap, detergents and disinfectants) to clean their

Table 3. Antibiotics used in this study to test for resistance/sensitivity.

Antibiotic	Abbreviation	Antibiotic disc conc. (µg)	Inhibition zone diameter (mm)		
			R	I	S
Cephoxitin	CX	30	≤21	_	≥22
Chloramphenicol	C	30	**≤12**	**13-17**	**≥18**
Ciprofloxacin	CIP	5	≤15	16-20	≥21
Erythromycin	E	15	≤13	14-22	≥23
Gentamycin	GEN	10	**≤12**	**13-14**	**≥15**
Penicillin	P	10	**≤28**	_	**≥29**
Tetracycline	TE	30	≤14	15-18	≥19
Trimethoprim-sulfamethoxazole	SXT	25	≤10	11-15	≥16
Vancomycin	VA	30	≤14	_	≥15

Source: The inhibition zone measurements were according to the Clinical and Laboratory Standards Institute 2011. The abbreviations are as they appeared on the antibiotic discs. R: - Resistance, I: - Intermediate and S: - Susceptible

hands. However, 2.3% of them used detergent to wash their hands.

Cross-contamination can be avoided if hand of milkers, utensils or equipment is washed with detergents and water in between using it after milking each cow. Hand washing is an essential component of infection control (Larson et al., 2003). During this study, it was observed that, majority of milkers (79.8%) did not wash their hands after milking each cow, but all of them washed their hands at the beginning. While 82% of milking utensils for each cow were not cleaned. Observations showed that some utensils were not cleaned properly, which could result in milk contamination due to microbes (*S. aureus*). The utensils in which the food is displayed for sale must be kept clean, covered and protected as they easily become contaminated if left dirty or unprotected (FAO, 2001).

Further observations revealed that most of the milkers had washed teats and udder before milking, but washing quality varied significantly. From food hygiene point of view, the quality of the working environment depends on the facilities or equipment provided like disposal of waste products. Based on observation, 82% of the lactating cows are housed in poor hygienic conditions.

Antibiotic susceptibility of *S. aureus*

The *S. aureus* isolates were tested for antibiotic susceptibility against nine antibiotic agents and they were classified depending on their inhibition zone diameter (Table 3).

Out of 98 isolates of *S. aureus*, antibiotic susceptibility tests were performed on 50 isolates. In this study, *S. aureus* isolates were found to be highly susceptible to ciprofloxacin (100%) followed by gentamycin (96%), vancomycin (82%), chloramphenicol (74%), erythromycin (68%), trimethoprim-sulfamethoxazole (66%), tetracycline (60%), cephoxitin (38%) and penicillin (6%). However, resistance to penicillin was most common (94%), followed by cephoxitin (62%), tetracycline (34%), trimethoprim-sulfamethoxazole (30%), chloramphenicol and erythromycin (20%), vancomycin (18%) and gentamicin (4%). All isolates tested for antibiotic sensitivity were susceptible to ciprofloxacin. The resistance pattern of *S. aureus* isolates to nine antibiotics tested in this study is shown in Table 4.

The highest resistance observed against penicillin in the present study is similar to that of Thaker et al. (2013) in India, which indicated as the overall (100%) *S. aureus* isolates were resistant to Penicillin-G. In Jimma Town, Ethiopia; Tariku et al. (2011) reported that out of 86 isolates of *S. aureus* which were isolated from Dairy farms, 87.2% were Penicillin resistant. Furthermore**,** Abera et al. (2010) in Adama Town, Ethiopia, showed that 94.4% of *S. aureus* isolates were resistant to penicillin.

The present study demonstrated that the resistant isolates may have been transferred to cow then to milk, which can be the reason of infection in human beings consuming raw milk. This contamination can be evaded by improving hygienic conditions and careful handling of cow during milking.

In this study, most of the isolates (96%; n=48) were resistant to one or more antibiotic agent. Five isolates (10.4%) were resistant to single antibiotic and 15 isolates (31.3%) showed resistance to 2 antibiotics. Multiple resistances to 3 or more antibiotics were found in 28 (58.3%) of *S. aureus* isolates (Table 5). However, 2 (4%) of isolates were sensitive to all antibiotic agents tested. The highest multiple drug resistance in *S. aureus* isolates were seen against three common antibiotics; Penicillin, Cephoxitin and Trimethoprim- sulfamethoxazole.

The overall susceptibility of *S. aureus* to all antimicrobial agents tested is resulted 288 (64%) susceptible, 14 (3%) intermediate resistant and 148 (33%) resistant (Figure 2).

The overall susceptibility (64%) of *S. aureus* isolates to

Table 4. Antibiotic sensitivity pattern of *S. aureus* (n=50) from lactating cow milk samples in Bahir Dar dairy farms, number (%).

Antimicrobial agent	Susceptible	Intermediate	Resistant
Erythromycin	34 (68)	6 (12)	10 (20)
Penicillin	3 (6)	-	47 (94)
Gentamicin	48 (96)	-	2 (4)
Trimethoprim- sulfamethoxazole	33 (66)	2 (4)	15 (30)
Chloramphenical	37 (74)	3 (6)	10 (20)
Vancomycin	41 (82)	-	9 (18)
Tetracycline	30 (60)	3 (6)	17 (34)
Cephoxitin	19 (38)	-	31 (62)
Ciprofloxacin	50 (100)	0 (0)	0 (0)

Table 5. Pattern of antibiotic resistance in bacterial isolates from lactating cow milk.

Number of antibiotics	Number of resistant isolates (%)	Resistance pattern
1	5 (10.4)	P (4) CX(1)
2	15 (31.3)	VA, P (2) TE, P (3) SXT, P (2) CX,P (6) E,P (1) P, CX (1)
3	15(31.3)	SXT,P, CX (7) TE, P, CX (2) C, TE, P (1) TE, VA, P (1)
4	6 (12.5)	P, E, CX (3) TE, P, CX (1) C, TE, P, CX (1) C, TE, VA, P (1) C, SXT, P, E (1) SXT,VA,P,CX(1) TE, SXT, P, E(1)
5	6 (12.5)	TE, SXT, P,CX (1) C, TE, VA, P, CX (2) C, TE, SXT, P, CX (2) C, VA, P, E, CX (1) TE, VA, P, GEN,CX(1)
6	1 (2)	C,VA, P, GEN, E, CX (1)

Total number of isolates = 48; values in parenthesis indicate percentage of the total isolates; C, chloramphenicol; CX, cephoxitin; E, erythromycin; GEN, gentamycin; P, penicillin; TE, tetracycline; SXT, trimethoprim- sulfamethoxazole; VA, vancomycin

all antibiotics tested in this study is in agreement with the reports of Mekonnen et al. (2005) in Ethiopia which shows isolates are 62.7% .The overall susceptibility in the present study is higher compared to that of Sasidharan et al. (2011) in Malaysia which is 6% to all antibiotics used. The present study has demonstrated the existence of high levels of resistance of *S. aureus* to commonly used antibiotics and the results are in accordance with reports from earlier studies in other countries which suggests that a possible development of resistance due to prolonged

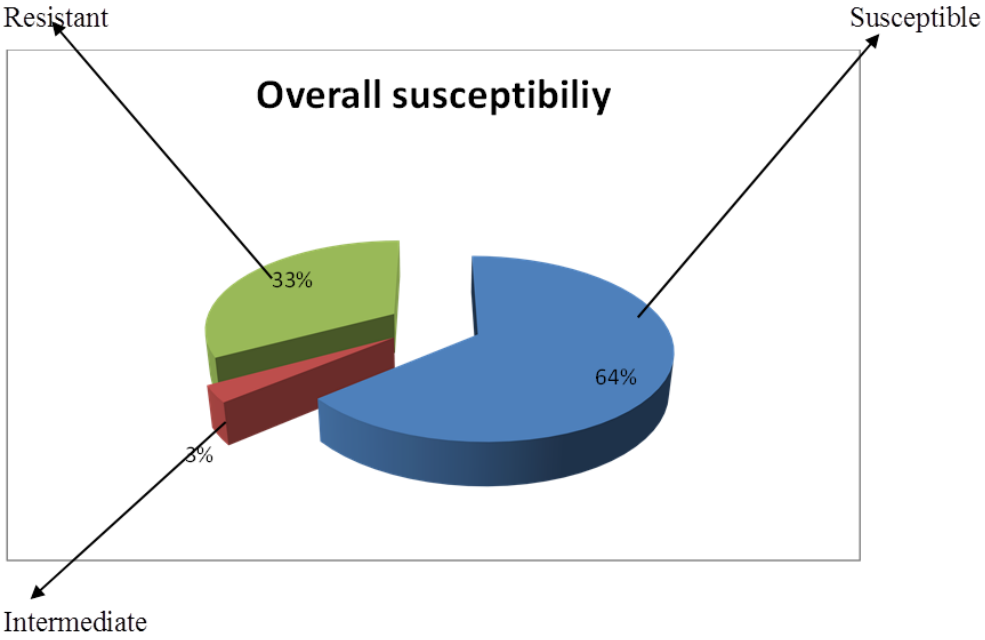

Figure 2. The overall susceptibility of *S. aureus* to all antimicrobial agents.

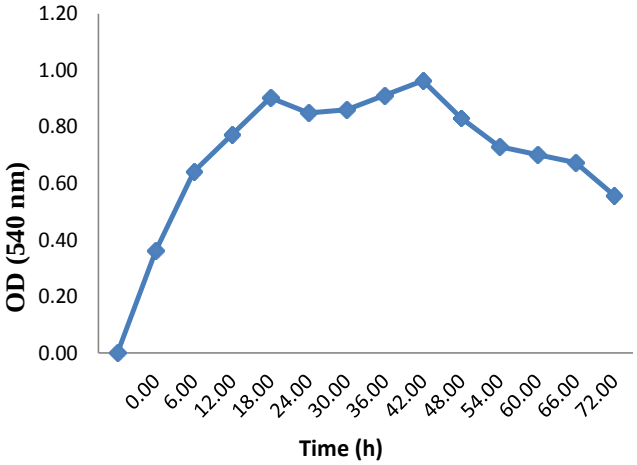

Figure 3. Growth curve of the most resistant *S. aureus* isolate.

and indiscriminate usage of antibiotics (Edward et al., 2002; Gentilini, 2000).

Growth pattern of *S. aureus* bacteria

The most resistant *S. aureus* isolate was used to assess the growth pattern in liquid medium (nutrient broth). Growth curve of the bacterial isolate indicated that *S. aureus* population increases linearly and sustains longer in fluctuating stationary phase (Figure 3).

S. aureus is a major pathogen of increasing importance due to the rise in antibiotic resistance. The growth and survival of *S. aureus* is dependent on the cells ability to adapt to environmental changes. *S. aureus* has evolved many mechanisms to overcome such changes, particularly in an infection (Lowy, 1998). A growth curve of *S. aureus* grown under ideal conditions can be divided into three phases: Lag, exponential, and stationary, as shown in Figure 3. During exponential phase, bacterium metabolism is rapid and efficiently to ensure constant growth. As the bacteria age and stop growing (post-exponential), cellular metabolism is re-organized for long-term survival under stationary conditions.

Conclusion

Results of the present study showed that lactating cow milk is highly contaminated by *S. aureus.* The study also revealed that *S. aureus* is still prevalent in small holder dairy farms in the study area. A large proportion of the isolates obtained in this study were resistant to three or more antibiotics. The presence of resistant *Staphylococci* in milk production poses a risk of spreading the pathogens to other animals, humans involved in animal care and milk processing, and consequently to the general population. The contamination of milk with *S. aureus* was associated with several risk factors. In general, the results of the present study revealed that milk provided to the consumers in the city was found less hygienic. Lack of general hygiene of milk handlers, personal hygiene, and environmental hygiene were identified as the major sanitary deficiencies. Therefore, the probability of milk contamination in these farms was high.

Recommendations

Based on the results of present study the following recommendations are forwarded:

1. Information on health hazards associated with contaminated raw milk should be extended to the public, so that consumption of untreated/improperly treated raw milk could be avoided.
2. Farmers should ensure strict personal hygiene and that of animals, and general sanitary condition of the farms should be improved and maintained.
3. Monitoring antimicrobial susceptibility in pathogenic bacteria in animals is recommended.
4. Further research is recommended about impacts and dynamics of genetic antibiotic determinants of *S. aureus*.
5. Proper risk assessment should be conducted to further clarify the possible health hazard for consumers related to the presence of *S. aureus* in milk.

Conflict of Interests

The authors have not declared any conflict of interests.

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APPENDIX

Observational check list

1. Description of the farm
Name _____
2. Herd composition
Species _____
3. Quality of milking environment
I) Floor hygiene: (a) relatively good_____
(b) Poor_____
4. Pre- milking hygienic condition of cows:
(I) Are teats and udder washed before milking? (a) Yes
(b) No
(II) Do you use any agent (detergent or disinfectant) for cleaning? a) Yes b) No
(III) Udder hygiene (a) Free of dirt_____
(b) Slightly dirty_____
5. Hand washing habit
I) Do milkers wash their hands before milking? (a) Yes
(b) No
II) Do milkers use detergent or disinfectant to clean their hands? a) Yes
(b) No
III) Have you drying your hands? (a) Yes
(b) No
IV) Do milkers wash their hands after milking each cow? (a) Yes
(b) No
6. Hair of milkers: Covered_____
Not covered_____
7. Milking utensils and environment
I) are milking utensils cleaned? (a) Yes
(b) No
II) Cleaning quality of milking utensils: (a) good_____
(b) Poor_____

Appendix Table 1. Time course growth pattern of *S. aureus* bacteria by measuring optical density.

Time (h)	Optical density (at 540 nm wave length)
0	0.361
6	0.642
12	0.7725
18	0.903
24	0.849
30	0.860
36	0.9115
42	0.963
48	0.831
54	0.730
60	0.7015
66	0.673
72	0.558

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Full Length Research Paper

Histamine and microbiological changes during storage of semi-preserved anchovies

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During the various stages of manufacture of semi-preserved anchovies, histamine shows a significant increase favored by the fragility of anchovies and wealth histidine, an amino acid precursor of histamine. The decarboxylation of histidine is present as a major problem in the industry of semi-preserved anchovies, especially during the ripening step. The objective of this work is to monitor the levels of alteration in a final product subjected to aging by incubation at 30°C ± 1. Accumulation of histamine, as well as microbial population in semi preserved anchovies were investigated. Enterobacteriaceae and Lactobacilli do not seem to resist to the salt applied to the product. They disappear within one month of storage. A total of 665 bacterial strains were selected from the prescreening step using various selective media. Only 20.6% of these selected isolates showed a positive reaction in Niven's differential medium, and 31.4% of the positive isolates were true histamine formers when confirmed by thin layer chromatographie. The values of histamine remain in close contact with the sanitary measures taken by each company. Regular monitoring and mastering of good practices are necessary for a good quality product.

Key words: Anchovy, bacteria, histamine, histidine, semi-preserved anchovies.

INTRODUCTION

Biogenic amine are basic nitrogenous compounds occurring in meat, fish, cheese and wine products, mainly due to amino acid decarboxylation activities of certain microbes (Hungerford, 2010). Histamine is a biogenic amine produced by decarboxylation of free histidine. Histamine is normally present at low levels in the human body, can be present in a variety of foods such as fish, cheese, meat, wine, and fermented foods. Since histamine is involved as a primary mediator in many allergic reactions, the increase in its levels to values greater than 500 mg/kg can be highly toxic giving symptoms that can be confused with alimentary allergies (Baross et al., 1992).

Histamine content in fish can rapidly increase during spoilage by bacterial histidine decarboxylases. High levels of histamine content have been found in various types of fish implicated in scombroid poisoning (Hwi-Chang Chen et al., 2008). A Scombroid fish (Teleostea,

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Scombroidei), such as mackerel and tuna, or clupeid fish (Teleostea, Clupeoidei) such as sardines, anchovies and herring, are frequently involved in histamine toxicity. These fishes have relatively high free histidine levels in their muscles when alive (Hernández-Herrero et al., 1999). Post-mortem proteolysis liberates additional histidine from muscle protein, and explains why histamine can reach high concentrations without the formation of organoleptic spoilage indicators. Significant relationship was observed between histamine content and Enterobacteriaceae count. Enterobacteriaceae species are the most important histamine-forming bacteria in tuna fish (Koohdar et al., 2011).

Anchovies, in brine or in oil, are semipreserved, obtained without any heating process to stabilize the final product. Changes during storage can occur, and even at low temperatures, enzymatic processes may lead to a product showing a high degree of proteolysis. Moreover, desalting and filleting increase the risk of secondary bacterial contamination in the final product packed immersed in oil. Previous studies showed that histamine levels increased during storage of semipreserved anchovies when they were not kept at refrigeration temperature (Yung-hsiang et al., 2005). Other biogenic amines such as tyramine, â-phenylethylamine, and tryptamine also seem to increase during storage (Veciana-Nogue's et al., 1997), but little information is available.

The preparation of semi-preserved anchovies *Engraulis encrasicholus* is a process of salting and ripening, (Hernández-Herrero et al., 1999). The ripening process is common in some Mediterranean areas and Argentina, where the species is *Engraulis anchoita*, (Triqui and Reineccius, 1995). Anchovy (*E. encrasicholus*) is primarily marketed and processed in Spain, Italy, Greece, France, and Morocco, (Hernández-Herrero et al., 1999).

The production of anchovy fillets for subsequent canning is a delicate job, usually requiring female hands. The process begins with salting and pressing of the fish which are then left to cure for several months until they acquire the right reddish color and aroma. Penetration of salt varies with the thickness of the muscle, temperature, the freshness and the fat content, (Clucas, 1982). After that, the fish are skinned, washed, trimmed to size then dried and filleted. Finally, they are placed in containers (cans or glass jars), and covered with olive oil before sealing. Unlike other preserves, in neither case are anchovies subjected to sterilization because heat would spoil them. This is why they are treated as semi-preserves. Cans must be stored at between 4 and 12°C, and the contents should be consumed between six months and one year after production.

Here we studied the evolution of the contents of histamine throughout the shelf life of ripened anchovies from the Morrocan coast. Changes in histamine during storage of anchovies packed immersed in oil and ready to eat from three different manufacturers were studied during 12 months of storage of the final products and stored under two temperatures, 4 and 30°C ± 1. The assigned shelf-life period for these anchovies is 6 months. Total mesophilic, halophilic, lactic flora and enterobacterial counts were also determined to check their possible relationship with histamine production linked to the handling process needed before the final packaging of anchovies immersed in oil.

Table 1. Characterization of samples used.

Company	Description	Packaging
S1	Fillet anchovy caper rolled	Canned product
S2	Anchovy fillet	Glass jars
S3	Anchovy fillet	Canned product

MATERIALS AND METHODS

Sampling

Samples of semi-preserved anchovies are from three different companies S1, S2 and S3 (Table 1). Anchovies are caught in the coast of Morocco and prepared in the same way (heading and gutting, ripening, washing, threading, oil immersion and packaging). Samples of each company were incubated at two temperatures, 4°C ± 1 in a refrigerator (SANYO MPR 311D) as recommended storage temperature of the final product, and 30°C ± 1 in an incubator (SANYO MCO 175), which is the temperature that causes aging of the product. Histamine departing values are 3, 12 and 70 ppm for the S1, S2 and S3 companies. Each month, samples are taken for analysis, a mixture of five cans to determine the histamine content and perform a bacterial count.

Chemical analysis

The histamine assay is done according to the spectrofluorometric method of Lerk and Bell (1978). 10 g of sample is homogenized in 90 ml of 10% trichloroacetic acid filtered buffer. 200 µl of the filtrate is transferred with 150 ml of acetate buffer in an ion exchange column. Histamine is then eluted with hydrochloric acid. Reading the DO at excitation wavelength of 350 nm and emission wavelength of 450 nm after complexation of 20 µl filtrate with Ortho-phthaldehyde. To measure the pH, a sample of 10 g is homogenized in 10 ml of distilled water and the pH is measured using a Toa DKK pH meter HM- 20J.

Microbiological analysis

From a mixture of 5 can of each company, 25 g were removed aseptically and homogenized with 225 ml of buffered peptone water in a stomacher bag for a count of the total flora of PCA medium (Plate Count Agar) at 30°C ± 1 for 72 h, the total halophilic flora in PCA medium based on sea water at 25°C for 5 days (SANYO MIK-153). Sea water is filtered before to a porosity of 0.45 µ. Enterobacteriaceae on VRBG medium (Glucose Agar with Crystal Violet, the Red neutral and Bile) at 30°C ± 1 for 24 h (ISUZU FR-114S). The lactic flora in MRS media (De Man, Rogosa and Sharpe) and M17 respectively bacilli and shells at 30°C ± 1 for 48 h (ISUZU FR- 115S). Seeding is double layer for VRBG, MRS and M17.

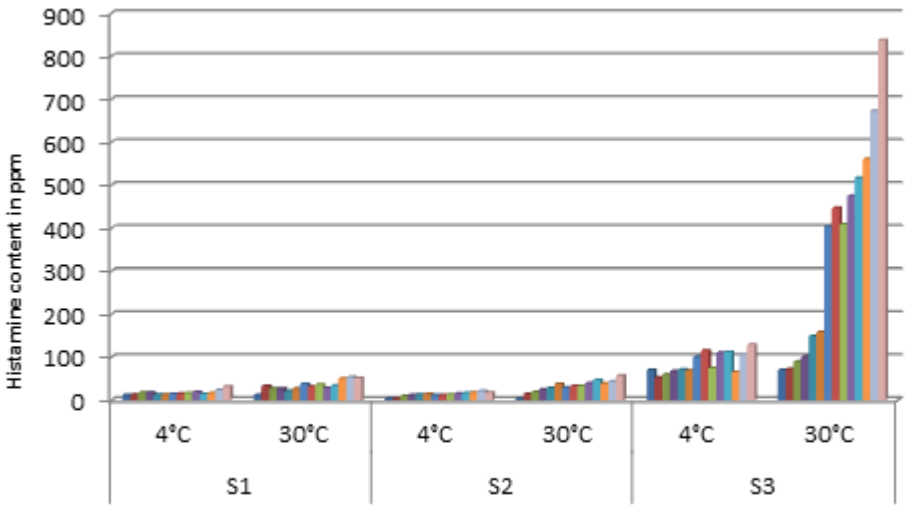

Figure 1. Influence of storage temperature on the histamine formation in semi preserved anchovies

To check the hability of bacteria to produce histamine, 665 strains were isolated and tested on Niven medium (L- histidine 2 HCl 27%, 5% tryptone, 5% yeast extract, sodium chloride 5%, 1% Calcium Carbonate, Agar 30%, 0.6% bromocresol purple). and Yamani and Unterman medium (2% peptone, Lab Lemco powder 1%, 5% sodium chloride, L- histidine HCl 10%, 0.1 % bromocresol green, chlorophenol red 0.2%). The incubation is done at 30°C ± 1 for 24 h (ISUZU FR- 114S) strains are considered positive if they have a blue purple halo on agar Niven, or broth turn from green to blue in the Yamani & Unterman medium.

RESULTS

Histamine content recorded during the storage of the final product evolves differently for the three companies. The company S3 displays the highest values exceeding 800 ppm after one year of storage at 30°C ± 1. However, histamine values stored at 4°C ± 1 increase lower than samples kept at 30°C ± 1. Therefore, we would point out that storing semi preserved anchovies under refrige-ration, as recommended, reduces but does not prevent histamine formation.

Both S1 and S2 companies have similar histamine values and remain low compared to standards. The highest and earliest formation of histamine was observed in samples from S3 (Figure 1). Histamine contents after 6 months of storage at 30°C ± 1 surpassed the maximum average value (200 ppm) permitted for ripened fish product. Histamine levels remained constant in S1 and S2 samples stored at 4°C ± 1 and increase at 30°C ± 1 were, in general, lower than recommendation.

The average bacterial count in three companies studied is almost similar in the three culture media PCA, PCA in sea water and M17. There was a slight increase during the first two months which exceeded 10^6 cfu/g, followed by a gradual decline until the end of the storage period (Figure 2). Note however that counts of bacteria belonging to the *Enterobacteriaceae* family on VRBG and Lactococcus on MRS media disappear after the first month of storage. Most frequently isolated profilic histamine-formers fish under controlled storage conditions are mesophilic enteric bacteria such as *Morganella morganii*, *Proteus vulgaris*, *Hafnia alvei* and *Citrobacter freundii* (Chiara et al., 2012).

When the Niven, Yamani and Unterman medium was used, only 20.6% strains were found to be positive and forming histamine. The majority of these bacteria is represented by the *Enterobacteriaceae*, halophilic total flora, and lactic bacteria (Figure 3).

DISCUSSION

Biogenic amines are formed mainly through decarboxylation of specific free amino acids by exogenous decarboxylases released by the microbial species associated with seafood. Many bacterial species are known to possess histidine decarboxylase and have the ability to produce histamine (Yi-Chen Lee et al., 2016). Histamine formation in fish is related to the environmental conditions, histidine content, and the presence of histamine forming bacteria. Traditionally, histamine formation in fish has primarily been prevented by limiting microbial growth with chilling and freezing (Jia-Wei et al., 2015). High levels of histamine can induce symptoms similar to allergic reactions and ingestion 100-1000 mg once can cause histamine poisoning, (Rossano et al., 2006).

It seems well established that histamine formation increases with temperature during storage, but remains dependent on the initial quality of the final product. The results indicate that both S1 and S2 record small

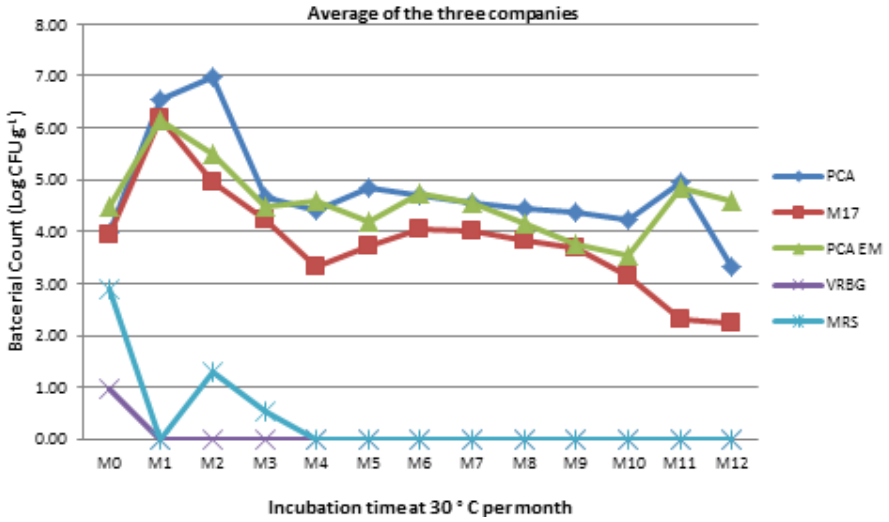

Figure 2. Average bacterial flora during the incubation of semi preserved anchovy at 30°C ± 1.

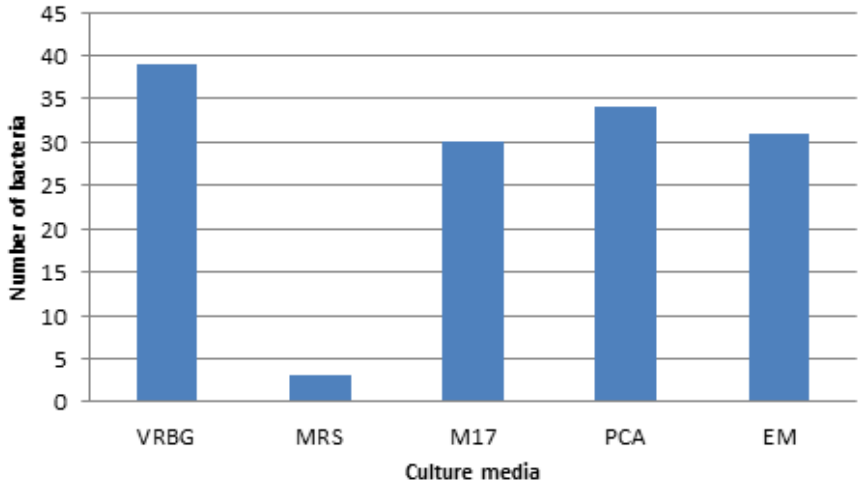

Figure 3. Histamine forming bacteria from semi preserved anchovies incubated at 30°C ± 1 isolated in different culture media.

increases histamine content even at storage of 30°C ± 1, 52.03 and 57.65 ppm respectively. However, the S3 show a high level on histamine 837.93 ppm knowing that the initial value is 70.69 ppm. Low temperature delayed the growth of bacteria, resulting in minimal changes of histamine.

High levels in histamine in anchovies, even before the shelf life, may be related to certain toxic effects. The dose of 10 mg histamine was related to symptoms of histamine poisoning, (Veciana-Nogue's et al., 1997). Anchovies are usually consumed in small quantities as a food ingredient in many dishes, and it can be difficult to surpass the levels previously mentioned.

However, taking into account the weight of 60 g anchovy in a final product, the histamine content recorded in all these stored samples at 4°C ± 1 for one year remains insufficient either to cause histamine poisoning or headache. After 12 months of storage at 30°C ± 1, the consumption of 12 g of the anchovy from S3 could provide more than 10 mg of histamine. Both S1 and S2 companies stay away from the dose adverse effect. The histamine toxicitiy level was exceeded in S3 after only 6 months of storage at 30°C ± 1.

Conclusion

The results of this study show that the semi-preserved anchovies deteriorate easily and can be transformed into dangerous products during storage, especially if the store does not meet the conditions recommended by the

manufacturer. The fragility of the anchovies and persistence of histamine-forming bacteria in the product contribute significantly in this alteration. Good control of the manufacturing process and good hygiene practices is needed to prevent the introduction of Enterobacteria in the product at any stage of its manufacturing.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Molecular genotypes of *Mycobacterium tuberculosis* strains circulating in Dakar, Senegal

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Tuberculosis is a contagious infectious disease, in which epidemiologic monitoring by molecular approaches is a critical element of prevention and control. The population structure of the *Mycobacterium tuberculosis* complex (MTBc) in Senegal was last described in the 1970's using biochemical methods. In this present study, we applied molecular approaches to genotype *M. tuberculosis* isolates from active pulmonary tuberculosis patients who participated in a prospective cohort study between 2004 to 2006. Genetic characterization, using standard spoligotype analysis and Line Probe Assay for resistance to isoniazid and rifampicin, was applied after culture on egg based solid media. The prevalence of resistance to isoniazid was 1.0% and to rifampicin 0.0% among 203 isolates tested. Of the 203 isolates, spoligotype patterns present in the TB-insight database were identified in 178 (87.6%) while 25 (12.3%) showed patterns suggestive of mixed infection. The major spoligotypes identified were the Haarlem lineage (22%), followed by the T (19%), Beijing (12%), LAM (12%), and *M. africanum* West African 2 (10%). Patterns suggestive of mixed infections, such as the sole lack of spacers 33 and 34, suggested a combination of Euro-American *M. tuberculosis* and Beijing lineage, which were confirmed by polymerase chain reactions (PCRs) for lineage defining deletions in a subset of isolates. The population structure of the *M. tuberculosis* complex in Dakar reflects a predominance of Euro-American *M. tuberculosis* (Haarlem, T and LAM), with a decreased prevalence of *M. africanum* West African 2, compared with reports from the 1970's based on biochemical speciation, which reported prevalence of *M. africanum* around 20% in Dakar.

Key words: Tuberculosis, *Mycobacterium tuberculosis* complex, polymerase chain reactions (PCRs).

INTRODUCTION

Tuberculosis (TB) remains a major cause of illness and death worldwide, especially in Africa and Asia. In 2014, the National Tuberculosis Program (NTP) reported 13.647 new TB cases in Senegal, of which 7% were co-

infected with HIV. With a population of about 14 million inhabitants, TB incidence is estimated at 138 cases per 100,000 persons per year (all forms), and prevalence at 205 cases per 100,000 persons (WHO, 2015). Molecular genotyping tools for *Mycobacterium tuberculosis* have become valuable for TB diagnostics and investigations of disease transmission dynamics, outbreaks and phylogeny**.** They allow the distinction between "modern" MTBC lineages versus "ancient" lineages and their respective geographical distribution worldwide.

Although these DNA typing techniques in conjunction with classical epidemiology approaches greatly enhance the understanding of TB transmission dynamics and epidemiology, no such study has been recently performed in Senegal. The only report that characterized circulating strains in the country was conducted by Niang et al. (1999). The authors presented preliminary work on 69 isolates using spoligotyping as a molecular tool to have an overview of strains, circulating in Dakar. As the sample size was relatively small, we are presenting a more comprehensive analysis on the mycobacterial population structure in Dakar between 2004 to 2006 using, molecular genotyping.

MATERIALS AND METHODS

Patients

The study population consisted of consecutive consenting new TB patients older than 18 years, who were part of a cohort of the African Tuberculosis Vaccine (AFTBVAC) study between, 2004 and 2006. Patients were recruited based on TB index case and their potential contact within a family. All subjects were diagnosed with clinically and bacteriologically confirmed tuberculosis. After written informed consent, sputa were collected from patients by clinicians from the Pulmonology clinic at Fann Hospital, Dakar, Senegal. All enrolled patients were resident of Dakar or suburbs surrounding the capital. Demographic and clinical patient data including age, sex, HIV status and severity of disease on chest radiography were recorded. This study was approved by the Ethics Committee of the Ministry of Health, Senegal.

Laboratory analysis

Sputum examination and TB culture were done at the Bacteriology laboratory of Aristide Le Dantec University hospital in Dakar, Senegal. After staining with the Ziehl Neelsen method, sputa were decontaminated with N-acetyl cysteine-NaOH. Two hundred microliters of decontaminated sputa were inoculated on each of two glycerol Lowenstein Jensen (LJ) slopes. Cultures were incubated at 37°C for up to 8 weeks. Positive cultures were confirmed by Ziehl Neelsen smear microscopy and conventional biochemical tests. We used Niacin test, urease, niacin accumulation, p-nitrobenzoic acid (PNB) and p-nitro-alpha-acetylamino-beta-hydroxypropio phenome (NAP) for discrimination of the MTB complex from Mycobacteria other than tuberculosis (MOTT) (Rastogi et al., 1989). Confirmed MTBc were tested for their sensitivity to streptomycin (STR), Isoniazid (INH), Rifampicin (RIF) and Ethambutol (EMB) and isolates were stored in glycerol at -80°C (Canetti et al., 1963).

DNA isolation

Genomic DNA was extracted from TB positive cultures as described elsewhere (van Embden et al., 1993). After spectrophotometry, 10 ng of DNA were used for spoligotyping analysis.

Spoligotyping analysis

Spoligotyping was performed on genomic DNA using commercially available activated Biodine C membranes with the 43 synthetic oligonucleotides covalently bound to the membranes (Ocimum Biosolutions, Huda Techno Enclave Madhapur, India), according to standardized methods as described by Kamerbeek et al. (1997).

Data analysis

The spoligotyping hybridization patterns were converted into binary and octal formats and compared with, previously reported strains in the Spol DB4 database (Brudey et al., 2006). Binary spoligotypes were entered in SITVIT2 database. In 2013, SITVIT2 contained genotyping information of about 75.000 *M. tuberculosis* clinical isolates from different countries. In this database, SIT (Shared International Type) designates spoligotypes shared by two or more *M. tuberculosis* isolates, as opposed to "orphan" which, designates patterns reported for a single isolate.

Detection of Rifampicin and isoniazid resistance

Resistance of isolates to rifampicin and isoniazid was determined by the Genotype MTBDR*plus* Line Probe Assay (LPA) (Hain Lifescience GmbH, Nehren, Germany) according to the manufacturer's instructions (Godreuil et al., 2007).

Single colony plating

Of two suspected mixed strain obtained after spoligotyping were re-cultured into 7H9 broth media, further plated on 7H11 solid medium that incorporates an enzyme that, digests casein to enhance growth of fastidious M.tb, species in single discreet colonies. Singles colonies of interest based on the cell morphology on 7H11 were marked with a felt pen and given a new lab ID.

RESULTS

Study population

The study population of 218 patients included 155 men (71%) and 63 women (29%), out of which five patients

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(2.2%) tested HIV positive. The age of patients ranged from 18 to 74 years.

Spoligotyping and assignment of mycobacterial lineage and families

Isolates were collected from 218 consecutive smear-positive TB patients out of which 15 (6.8%) did not yield a spoligotype pattern and were confirmed to be MOTT, based on 16S RNA and hsp65 sequencing. Non tuberculosis mycobacteria identified were: *M. fortuitum* 8 (53%), *M. terrae* 1 (6%), *M. intracellulare* 2 (13%), *M. gordonae* 1(6%), *M. abcessus* 1(6%) and two others species which, cannot be fully identified (13%).

Among the remaining 203 *M. tuberculosis* complex isolates, we identified a total of 59 different spoligotypes patterns. The majority of isolates belonged to the Haarlem family (22%), showing the largest spoligotype diversity with 14 different patterns, followed by the T (19%), Beijing (12%), LAM (12%), *M. africanum* West Africa 2 (MAF2) (10%), Cameroon (6%), U (0.4%), EIA5 (1.9%) and X2 (0.9%) (Table 1). Interestingly, 25 isolates (12.3%) were variations of the Manu family.

MTBDR plus results

Of the 203 strains tested by the MTBDR *plus* assay, only two strains (1.0%) showed resistance to isoniazid, and none to rifampicin. Out of the two INH resistant strains, one was a Beijing isolate with an inhA mutation, and the other one was, an EAI isolate with a mutation in katG.

Single colony culture

Of two 'Manu' pattern isolates that were plated to single colonies, for one isolate (D0242) the 'daughter' colonies revealed separate patterns of Lineage 2 (Beijing) and Lineage 4 (Euro-American), while the other 'daughter' isolates remained identical to the 'mother' isolate (Table 2).

DISCUSSION

An interesting finding of the present study is the relatively high proportion (12%) of non-endemic Beijing strains. Such a finding is of general public health interest in Dakar, as Beijing strains are associated with drug resistance, rapid spread and great virulence when compared to other *M. tuberculosis* strains (Gomgnimbou et al., 2012).

Moreover, it was recently suggested that some Manu 2 spoligotypes (which we frequently found in our study), could be the consequence of a "mixed" pattern due to concomitant Beijing and Euro-American strains (the latter comprising H, LAM, X, and T lineages) (Viegas et al., 2010). Therefore, considering the amount of Manu patterns and the possibility of mixed infections in our study, the above mentioned prevalence of Beijing strains could be an underestimation and potentially even be higher than the estimated 12%. Even at 12% prevalence, Dakar already has the highest proportion of TB due to the Beijing family in West Africa. The only other region in West Africa with comparable (though lower numbers) of Beijing strains (10%) is Cotonou, Benin. This phenomenon was identified during the first molecular epidemiological survey in Benin (Affolabi et al., 2009) and later confirmed to be an outbreak of streptomycin resistant Beijing strains in the city (Affolabi et al., 2009). Therefore the high proportion of Beijing strains in 2004 to 2006 is concern in the monitoring of ongoing transmission amongst the current mycobacterial population and their resistance patterns, is important. So far our study suggests that antibiotic resistance was not a major concern in Dakar, as at the time of the study, only two isolates showed INH mono-resistance. Our molecular resistance testing suggests that drug resistance in new TB cases was low in the period of 2004 to 2006, with only 1% isoniazid resistance and no rifampicin resistance. An ongoing nationwide drug resistance survey, will allow us to compare these numbers with current drug resistance levels based on an unbiased sampling of, the entire population.

Our spoligotype results revealed a great diversity of the *M. tuberculosis* strains circulating in Dakar where we not only found the most common global spoligotypes (Brudey et al., 2006) but also the *M. africanum* West Africa 2 lineage, exclusively present in the region. Specifically, these include the Haarlem family followed by T, LAM, Beijing, and MAF2 families. Interestingly, we did not identify any *M. bovis* isolates, which is in line with a previous observation by Diop et al. from 1970 (Diop et al., 1976). The described composition of the MTBc overall confirms the findings from a study, analyzing 69 Senegalese *M. tuberculosis* isolates collected between September 1994 and September 1995, as comparable to our study and the major identified families were the same (Niang et al., 1999).

A major difference between the present and previous studies is in regards to the prevalence of MAF2 in Dakar. A total of 20% of all pulmonary tuberculosis cases in 1970 were still infected by MAF2 (Diop et al., 1976), indicating a slow decrease of the MAF prevalence in Dakar. The present study showed a 10% prevalence of MAF2 in tuberculosis patients between 2004 to 2006, which is similar to 10% in the study conducted in 1995. Countries such as Guinea Bissau, Benin and Nigeria observe high MAF prevalence up to 40% (Kallenius et al., 1999; Affolabi et al., 2009), while a decrease of *M. africanum* prevalence has been observed in other West and Central African countries like Cameroon and Burkina

Table 1. Shows the different Spoligotypes pattern identified by a SIT in the SITVIT database.

Spoligotype description [a]	Octal code	Major Lineage[b]	Family[c]	N° of isolates percentage (%)	Suspect strains	Mixed
□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	000000000003771	East Asian (Beijing)	Beijing	25 (12.31)	No	
□□□□□□□□□□□□□□□□□□□□□□□□□□□■□□□□□□■□□□□■■■■■■■	000000004020771	Euro-American	H2	1 (0.49)	No	
■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■	777777607760771	Euro-American	LAM	1 (0.49)	No	
■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□■□□□□■■■■■■■	777777774020771	Euro-American	H1	1 (0.49)	No	
■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■■■■■	777777777720771	Euro-American	H3	22 (10.83)	No	
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	777777777760771	Euro-American	T	24 (11.82)	No	
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□■■■■■■■■■	777777777763771	Indo-Oceanic	Manu2	4 (1.97)	Yes	
■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■□□□□■■■□■■■	777777607760731	Euro-American	LAM4	16 (7.88)	No	
■■■■■■■■■■■■■■■■■■■■■□□□■■■■■■■□□□□■■■■■■■	777777743760771	Euro-American	Cameroon	13 (6.4)	No	
■■■■■■■■■■■■■■■■■■■■■■□□□□□□■□□□□■■■□■■■	777777774020731	Euro-American	H1	2 (0.98)	No	
■■■■■■■■■■■□□□□■■■■■■■■■■■■■■■□□□□■■■■■■■	777703777760771	Euro-American	T	5 (2.46)	No	
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□■■■■■	777777777700771	Euro-American	T	1 (0.49)	No	
■■■■■■■■■■■■■■■■□■■■■■■■■■■■■■□□□□■■□□□□■	777776777760601	Euro-American	X2	1 (0.49)	No	
■■■■■■□□□■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■■■■	770777777777671	West African 2	AFRI_2	21 (10.34)	No	
■■■■■■■■■■■■■■■■■■□□□□□□■□□□□□□■□□□□■■■■■■■	777777004020771	Euro-American	H1	4 (1.97)	No	
■■■■■■■■■■■■■■■■■□■■■■■■■■■■□■□□□□■■■■■■■	777777377720771	Euro-American	H3	4 (1.97)	No	
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□□□■	777777777760601	Euro-American	T	1 (0.49)	No	
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□■■■■	777777777760671	Euro-American	T	1 (0.49)	No	
■■■■■■■■■■■■■■■■■■■□■■■■■■■■■■□□□□■■■■■■■	777777677760771	Euro-American	T	1 (0.49)	No	
■■■■■■■■■■■■■■■■■■■■■□□□□□□■□□□□■■■□■■■	777777770020731	Euro-American	H1	1 (0.49)	No	
■■□■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■■■■■	677777777413771	Indo-Oceanic	EAI	1 (0.49)	No	
■■■■■■■■■■■■■□■■■■■■■■■■■■■■■■□□□□■■■■■■■■	777757777760771	Euro-American	T	1 (0.49)	No	
■■■■■■■■■■□□□□■■■■■■■■□■■■■■■■■□□□□□□□□□□□□□	777417737700000	Euro-American	X2	1 (0.49)	No	
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■□□■■	777777777720711	Euro-American	H3	1 (0.49)	No	
■■■■■■■■□□□■■■□□□■□□□□□□□□□□□□□□□□□□□□□■■■■	776161000000071	Indo-Oceanic	U	1 (0.49)	No	
■■□■■■■■■■■■□■■■■■■■□□□□■■■■■■■■□□□□■■■■□■■	677737607760751	Euro-American	LAM2	1 (0.49)	No	
■■■■■■■■■■■■■■■■■■■■■■□■■■■■■□■□□□□■■■■■■■	777777767720771	Euro-American	H3	1 (0.49)	No	
■■■■■■■■■■■■□■■■■■■■■■■■■■■■■□■□□□□■■■■■■■	777757777720771	Euro-American	H3	4 (1.97)	No	
■■■■■■■■■■■□■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■	777677607760771	Euro-American	LAM	1 (0.49)	No	
■■□□■■■■■■■■■■■■■□■■■■■■■■■■□■□□□□■■■■■■■	637777377720771	Euro-American	H3	1 (0.49)	No	
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□■■■	777777777760631	Euro-American	T	1 (0.49)	No	
■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■□□■■■■■■■■■	777777607763771	Indo-Oceanic	Manu2	2 (0.98)	Yes	
■■■■■■■□■■■■■■■■■■■■■■■■■■■■□■□□□□■■■■■■■	776777777720771	Euro-American	H3	1 (0.49)	No	
■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□■□□□□■■■■□■■	777777774020751	Euro-American	H1	1 (0.49)	No	
■■□□□■■■■■■■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■	617777607760771	Euro-American	LAM1	1 (0.49)	No	

Table 1. Contd.

■■■■■■■■□■■■■■■■■■■■□□□□■■□□□□□■□□□□■■■■■■■	776777606020771	Euro-American	LAM11-ZWE	1 (0.49)	No
■■■■■■■■■■■■■■■■■■■■■□□□■■■■■■■■■□□□□■■■■■■■	777777617760771	Euro-American	T	1 (0.49)	No
■■■■■■■■□■■■■■■■■■■■□□□□□■■□□□□■□□□□■■■■■■■	776777603020771	Euro-American	LAM11-ZWE	1 (0.49)	No
■■■■■■■■□□□□□□■■■■■■■□□□□■■□□□□□■□□□□■■■■■■■	776017606020771	Euro-American	LAM12-Madrid1	1 (0.49)	No
■■■■■■■□■□■■■■■■■■■■□□□□■■□□□□□■□□□□■■■■■■■	775377606020771	Euro-American	LAM11-ZWE	1 (0.49)	No
■■■■■■■■■■■■■■■■■■■■□□□□□■■■■■■■■□□□□■■■■■■■	775377606020771	Indo-Oceanic	Manu3	1 (0.49)	No
■■■■■■■■■■■■■□□■■■■■□□■■■■■■■■□■□□□□■■□■■■■	777747637720671	Euro-American	H3	1 (0.49)	No
■■■■■■■■■□■■■□□■■■■■□□□■■■■■■■■■□□□□□■■□□■■	777347617760311	Euro-American	T	1 (0.49)	No
■■■■■■■■□■■■■■■■■■■■□□□□□■■□□□□■□□□□■■■■■■■	776777603020771	Euro-American	LAM11-ZWE	1 (0.49)	No
■■■■■■■■■■□□□□□□□□□□□□□□■■■■■■■□■□□□□■■■■■■■	777400017720771	Euro-American	H	1 (0.49)	No
■■■■■■■■■■■■■□■■■■■□■□■■■■■■■■■□□■■■■■■■■■	777767657763771	Indo-Oceanic	Manu2	1 (0.49)	yes
■■■■■■■■■■■■■□□■■■■□□□■■■■■■■■■□□□□□■■□■■■	777763617760331	Euro-American	T	1 (0.49)	No
■■■■■■■■■■■■■■■■■■■□□■■■■■■■■■□□■■■■■■■■■	777777637763771	Indo-Oceanic	Manu2	1 (0.49)	yes
■■■■■■■■■■■■■■■■■■■□■□■■■■■■■■■□□■■■■■■■■	777777657771771	Indo-Oceanic	Manu3	1 (0.49)	yes
■■■■■■■■■■■■■■■■■■■■■□□■■■■■■■■□□■■■■■■■■■	777777747763771	Indo-Oceanic	Manu2	1 (0.49)	yes
■□□■■■■■■■■■■■■■□■■■■■■□□□□□□□□□□□□□□□□□□□	477776770000000	Indo-Oceanic	EAI3-IND	1 (0.49)	No
■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■□□□□■■■■■■■	777777703760771	Euro-American	T	1 (0.49)	No
■■■■■■■■■□■■■■□■■■■■■■■■■■■■■■■■□□■■■■□■■■■	777357777763671	Indo-Oceanic	Manu2	2 (0.98)	Yes
■□□■■■■■■■■■■■■■□■■■■■■□□□□□□□□□□□□□□□□□□□	477776770000000	Indo-Oceanic	EAI3-IND	1 (0.49)	No
■■■□□■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□□□■	717777777760601	Euro-American	T	1 (0.49)	No
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■■■■■■■■■■	777777777767771	Indo-Oceanic	Manu2	12 (5.91)	Yes
■■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■■□□■□■■■■■■■	777757777762771	Indo-Oceanic	Manu2	1 (0.49)	Yes
■■■■■■□□■□□□■□□■□■■■□□□■■□□□□□□□□□■■■■■■■■■	771045614003771	Indo-Oceanic	EAI	1 (0.49)	Yes

a: The black and white boxes indicate the presence and absence, respectively, of the specific spacer at positions 1-43 in the DR locus b: Major lineage according to SpolDB4 database c : Family designation according to SpolDB4 database.

Faso (Huet et al., 1971; Ledru et al., 1996; Godreuil et al., 2007; Affolabi et al., 2009; Gehre et al., 2013). However, latest studies described 20% MAF2 prevalence in Burkina and 9% of MAF1 in Cameroon (Niobe-Eyangoh et al., 2003; Gomgnimbou et al., 2012). This reduction could be due to the recent expansion of the so-called *M. tuberculosis* Cameroon family in the country (Niobe-Eyangoh et al., 2003). Interestingly, the Cameroon family was already found in a proportion of 6% in our study indicating that, this family is successfully spreading across West African countries including Senegal**.** The reason for the relative decline of *M. africanum* in West Africa could be due to a lower transmission capacity or lower virulence. Although the virulence capacity of MAF2 strains is not well known, it was already demonstrated that these bacteria are overall attenuated and less immunogenic than *M. tuberculosis* sensu stricto isolates (de Jong et al., 2006; Bold et al., 2012). Another reason for the decline of *M. africanum* in West Africa could be that, certain *M. tuberculosis* lineages possess advantages in their ability to generate secondary cases within a community, and therefore outgrow *M. africanum* in the community (Gehre et al., 2013). The main limitation of our study is that, the geographical sampling area was limited to the Greater Dakar area and therefore is not a representative of the entire country.

Table 2. Spacer analysis of mixed infection test from single colonies.

Original Lab ID	Spoligotype signature [a]	Octal group	Major Lineage[b]
D0242	■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■□□■■■■■■■■■	777 777 607 763 771	Indo-Oceanic
Single colony ID			
A	Blank		
B	Blank		
C	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	000 000 000 003 771	East Asian (Beijing)
D	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	777 777 777 760 771	Euro-American
E	■■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■□□□□■■■■■■■	777 767 777 760 771	Euro-American
F	Blank		
G	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	000 000 000 003 771	East Asian (Beijing)
H	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	000 000 000 003 771	East Asian (Beijing)
I	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■□■■■■	000 000 000 003 671	East Asian (Beijing)
J	Blank		
DNA Neg.Control2	Blank		

A to **J** distinct colonies that differ morphologically by size and pigmentation. a: The black and white boxes indicate the presence and absence, respectively, of the specific spacer at positions 1-43 in the DR locusb: Major lineage according to SpolDB4 database.

It is recommended that the sampling should be extended to remote areas of Senegal that would allow the establishment of the population structure of *M. tuberculosis* in the whole country and follow the prevalence of Beijing, Lam 10 Cam and MAF2 one decade after isolation of the strains described here. Moreover, the used spoligotyping technique provides a broad overview of genotype variability and circulate *M. tuberculosis* strains in a certain area, yet has a fairly low discriminatory power (Brudey et al., 2006) and needs to be associated with other high resolution techniques such as, the MIRU-VNTR typing or sequenced-based SNP analysis if, transmission dynamics of a potential Beijing outbreak are the focus of a future study.

Conclusion

The genetic variability of the *M. tuberculosis* complex in Dakar reflects a predominance of Euro-American *M. tuberculosis* (Haarlem, T and LAM) lineage with lower prevalence of *M. africanum* West African 2 than previously described. Further investigations focusing on a larger collection of strains covering Senegal nationwide are necessary to describe the population structure of these *M. tuberculosis* strains more accurately and monitor the development of the increased spread of Beijing strains in the country.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Some parasitic copepods of selected Teleost and Chondrichthyan fishes from the Tunisian gulfs

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The examination of 415 specimen belonging to twelve species of Teleostean fish and 339 specimen belonging to seven species of Chondrichthyan fish collected from the Tunisian coasts, it enabled us to identify 10 species of parasitic copepods: *Lernaeopoda galei*, *Lernaeocera lusci, Neobrachiella merluccii, Hatschekia mulli, Caligus elongatus, Caligus pageti, Neobrachiella mugilis, Clavellotis fallax, Clavelissa pagri* and *Clavelissa scombri*. The distribution of copepods identified, varies from one host species to another. *Merluccius merluccius* and *Liza saliens* present the important number of copepods species. The copepod *Hatschekia mulli* is the most abundant. The presence of *Lernaeopoda galei* on *Scyliorhinus canicula, Mustelus mustelus* and *Mustelus punctalatus* was reported for the first time in the southern banks of the Mediterranean.

Key words: Copepods, Teleost fish, Chondrichthyan fish, Tunisian coasts, parasitic indices.

INTRODUCTION

Copepods are common parasites of marine fishes and have been reported from a great range of depths (Boxshall, 1998). This group of ectoparasites exhibits an astounding variety of lifestyles, host associations and morphology, to the extent that their crustacean affinities may be obscured (Huys et al., 2007). More than 2000 species of copepods parasitize marine and freshwater fishes and most are ectoparasitic: they are found all over the external body surface of the host as well as in more sheltered microhabitats that are permanently directly connected to the external environment, including the external nares, the eyes, the oral and branchial cavities, the gills and the cloaca (Rosim et al., 2013). Many copepod parasites negatively affect the appearance and reduced production of species of economically important fish, both from the wild and fish farms, thus making them difficult to market (Aladatohun et al., 2013). It is important to mention that in addition to their impact on host demographics populations, parasites also influence the ecosystem processes that are diverse as competition, migration and speciation of the hosts (Kaouachi et al., 2010).

Copepods parasites have been studied extensively in the world and in Tunisian coast, where they have become pests of Teleost fish species of commercial importance (Kabata, 1958; 1984; Faliex and Morand, 1994; Sasal et

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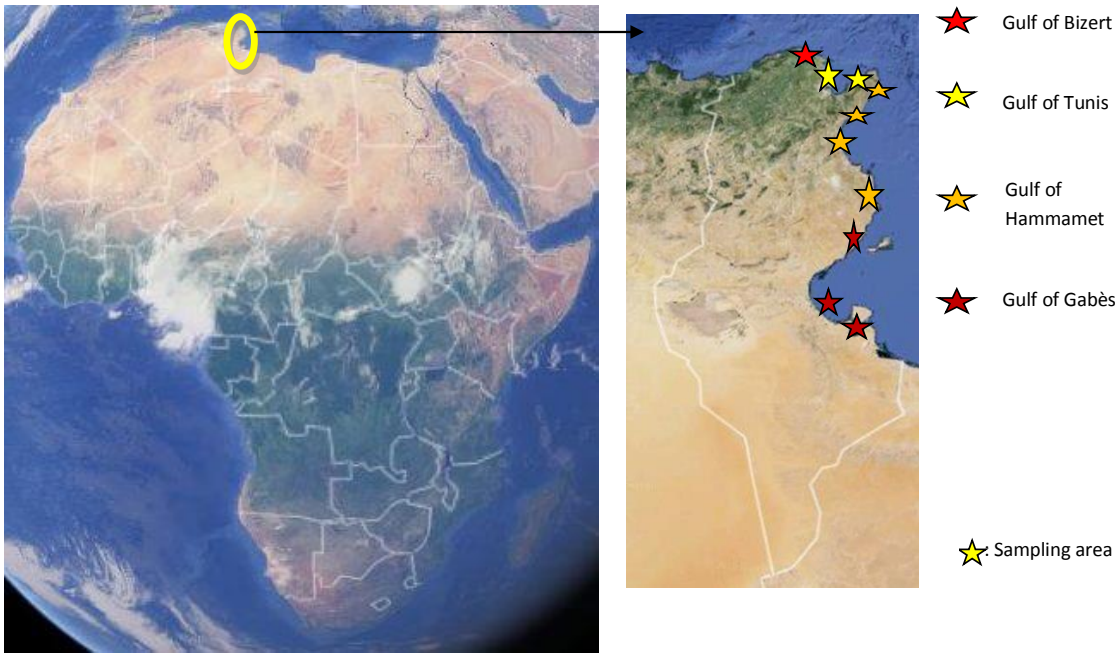

Figure 1. Location of the study.

al., 1996).

However, little is known of the diversity of parasitic copepods of Chondrichthyan fishes. The aim of this work is to analyze species of copepods parasitic richness, study copepods specificity and evaluate host epidiomogical characters by calculating infestation parameters. These features will be compared with other studies.

MATERIALS AND METHODS

A total of 415 specimens belonging to twelve species of Teleostean fish and 339 specimens belonging to seven species of Chondrichthyan fish were collected from 4 different Tunisian gulfs: Bizerte, Tunis, Hammamet and Gabès (Figure 1). The host species was identified using the method of Fischer et al. (1987) and Froese and Pauly (2014). Collected copepods were immediately removed from the hosts and preserved in 70% ethanol. Subsequently, specimens were cleared in lactic acid for 2 h prior to examination using stereo and light microscopy. Specimens were dissected on glass-slides and mounted as temporary preparations in lactophenol under a dissecting microscope. Measurements were made using an ocular micrometer. The drawings were made with the aid of a drawing tube. The data, the sampling area, the name and the size of host fish and the position of the parasite were noted.

Parasites species identification was based on morphological features according to Yamaguti (1963), Kabata (1979) and Ho and Kim (2004). The terms prevalence, mean intensity and abundance were used as defined by Margolis et al. (1982) and Bush et al. (1997) (Figure 1).

RESULTS

The examination of different host species allowed us to harvest 10 species of copepods. Among them are, 8 species which were present on Teleost fish (*Lernaeocera lusci, Neobrachiella Merluccii, Caligus pageti, Neobrachiella mugilis, Hatschekia mulli, Clavelissa scombri, Clavelloti spagri* and *Clavellotis fallax)* (Figures 3, 4, 5, 6, 7, 9, 10 and 11). On the other hand, only 2 species of copepods were collected on chondrichthyan fish (*Caligus elongatus* and *Lernaeopoda galei*) (Figures 2 and 8).

The hosts, the number of examined fish, the number of the infected fish, the parasitic indices (Prevalence (P), Intensity (I) and Abundance (A)) of each species of copepod were calculated, the specificity and the site of fixation are registered in Table 1.

Parasite spectrum

The examination of the whole fish species enabled us to collect 10 species of parasite copepods of which eight are hosted by Teleost fishes and two others are found in Chondrichthyan fishes (Table 1) (Figures 2 to 11).

Parasitic specificity

The study of parasitic specificity revealed that 3 of the species are O. Oixenous. However, 5 are *S. stenoxenous*. *C. elongatus* and *L. galei* are *E. Euryxenous* (Table 1).

Epidemiological characteristics of copepods species:

Our results (Table 1) show that, the distribution of

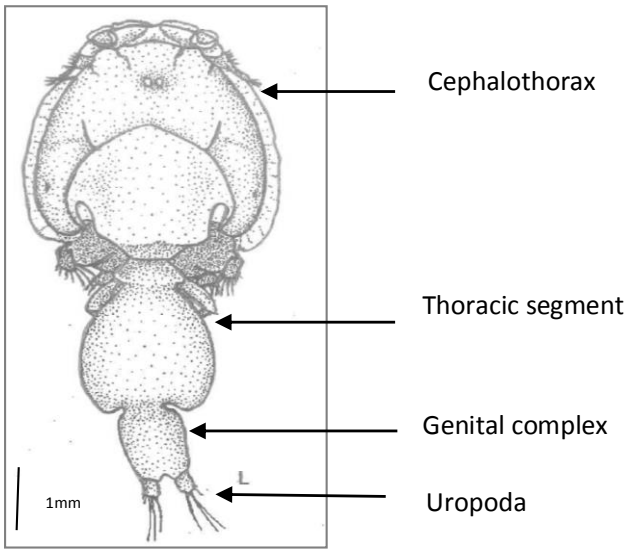

Figure 2. *Caligus elongatus* (Von Nordman, 1832) According to Kabata, 1979.

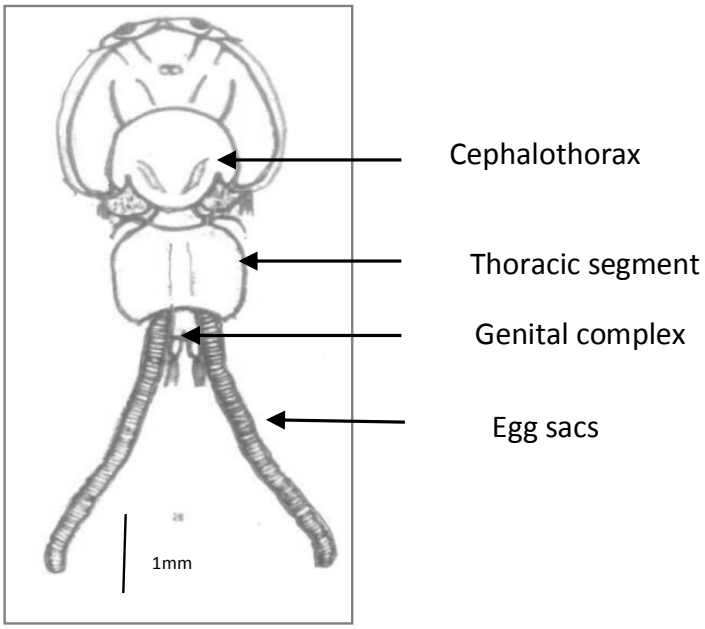

Figure 3. *Caligus pageti* (Russel, 1925) According to Ben Hassine 1971.

parasitological indices varies from one species to another. The important values of prevalence are observed in *M. barbatus* (P=29.3%), followed by *S. auratus* (P=13.33%), *Liza saliens* (P=10.8%) and *M. merluccius* (P=10%). However, all other copepods are less frequent and their prevalence is always lower than 10%. Furthermore, the maximum values of intensity is recorded in *S. scombrus* (I=1.5). *M. barbatus* shows the highest abundance (A= 0.29) (Figure 12 and Table 1).

L. galei was the only copepod collected on 3 different hosts (*S. canicula, M. mustelus* and *M. punctalatus*). We found the lowest prevalence in *M. punctalatus* (P= 3.04%) and the highest in *S. canicula* (P= 8.33 %). *L. galei* is more abundant in *S. canicula* (0.08) (Figure 13).

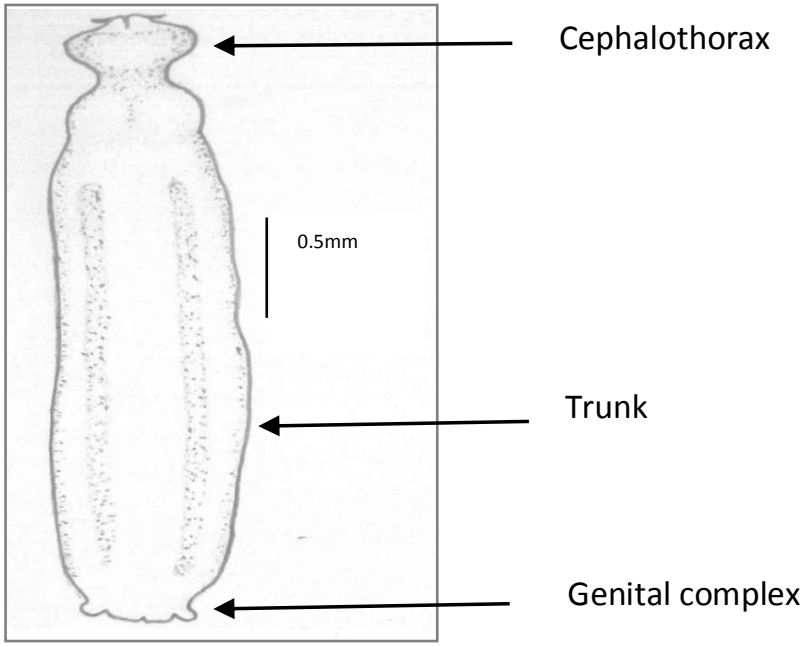

Figure 4. *Hatschekia mulli* (Van Benden, 1851) According to Kabata, 1979.

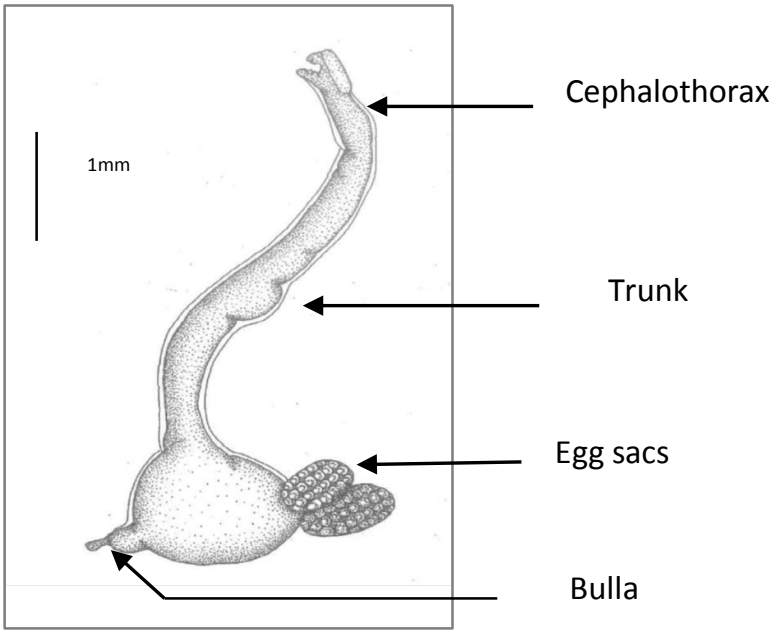

Figure 5. *Clavellisa scombri* (Kruz, 1877) According to Benmansour, 2001.

Analysis of richness of parasites per family

The analysis of the parasitic richness indicate that the family of Lernaeopodidae present the highest richness (MSR= 6) and the lowest one is recorded on Hatschekiidae (MSR=1) (Figure 14).

DISCUSSION

The observation of morpho-anatomical characters of copepod species, enabled the identification of 10 species (*C. elongatus, Caligus pageti, Clavelissa scombri, Clavellotis fallax, Clavellotis pagri, Hatschekia mulli,*

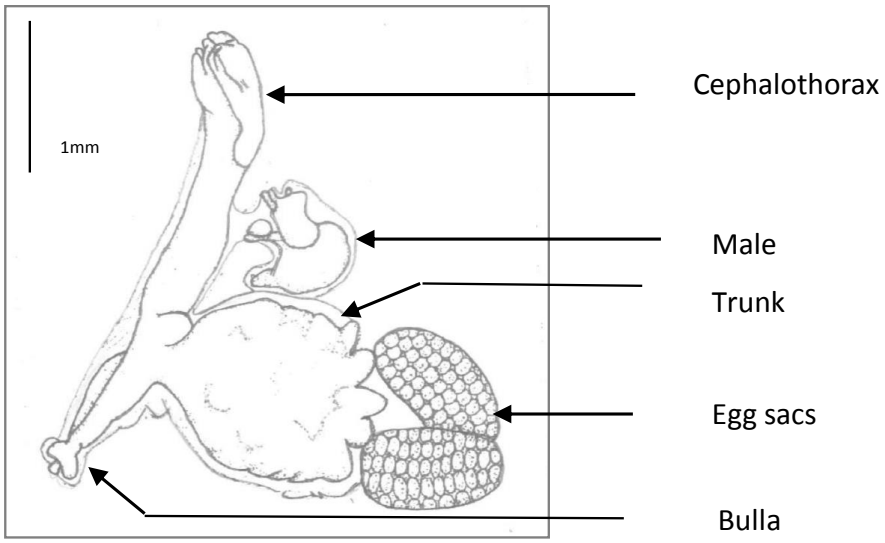

Figure 6. *Clavellotis fallax* (Female) (Heller, 1865) According to Benmansour, 2001.

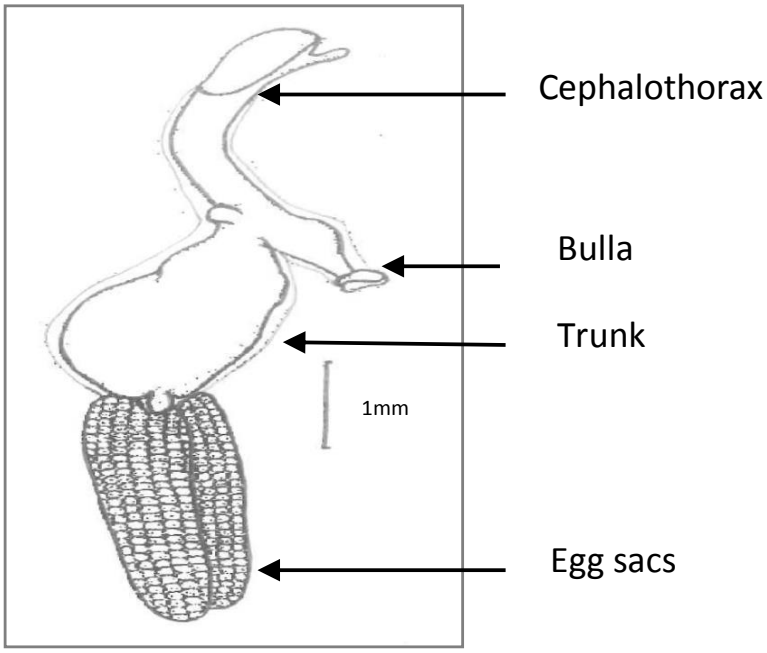

Figure 7. *Clavellotis pagri* (Kroyer, 1863) According to Benmansour, 2001.

Neobrachiella mugilis, Neobrachiella merlucii, Lernaeocera lusci and *L. galei*). In the Algerian coasts, *Clavellotis pagri* and *Hatschekia mulli* were also found in the gulf of Béjaia (Ramdane and Trilles, 2007) and the gulf of Annaba (Boualleg et al., 2010). The prevalence of *Clavellotis pagri* in our sampling is higher (P=9.67%) than the prevalence recorded in the Algerian coasts by Boualleg et al., (2010) (P=3.33%). Furthermore, the prevalence of *Hatschekia mulli* in tunisan coasts was more important (P=29.03%) than in Algerian waters (P=20.83%) (Boualleg et al., 2010).

Merluccius merluccius and *Liza saliens* present the highest number of parasitic species (2). In Tunisia, the highest parasitic diversity was mentioned by Benmansour and Ben Hassine (1997) in *Pagellus erythrinus* (6) and *Diplodus annularis* (5). On the coast of Algeria, *Pagellus erythrinus* and *Lithognathus mormyrus* present the important number of parasitic species (5) (Boualleg et al.,

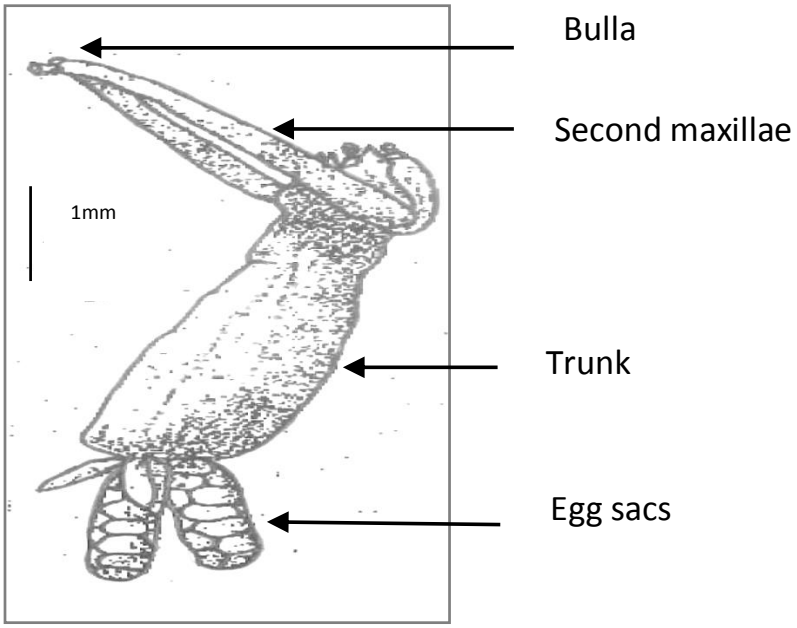

Figure 8. *Lernaeopoda galei* (Kabata, 1979) According to Radujkovic and Raibaut, 1987.

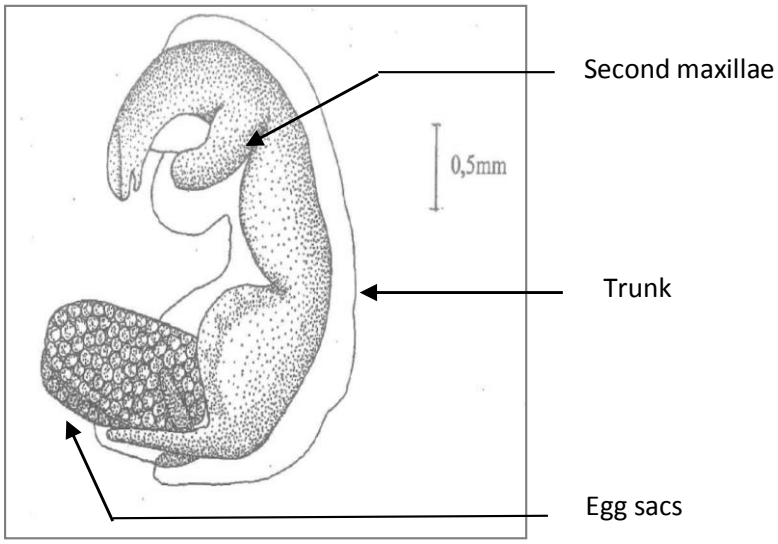

Figure 9. *Neobrachiella merluccii* (Bassett-Smith, 1896) According to Benmansour, 2001.

2010).

However, we found only two species of copepods on Chondrichthyan fish. The study of Essafi (1984) on Chondrichtyan fishes in Tunisian waters allowed in the collection of 22 different species of copepods. Nevertheless, we report for the first time the occurrence of *Lernaeopoda galei* on *Scyliorhinus canicula, Mustelus mustelus* and *Mustelus punctalatus* on the southern banks of the Mediterranean. This can be explained by the scarcity of studies on parasitic copepods of Chondrichthyan fish in this part of the Mediterranean. *L. galei* parasite in several Chondrichthyan fish species was never harvested on *M. punctalatus*. This is the first mention of this copepod on this host fish.

Also we assign *Caligus elongatus* as parasite of *Raja clavata* in Tunisia. *C. elongatus* parasitize several species

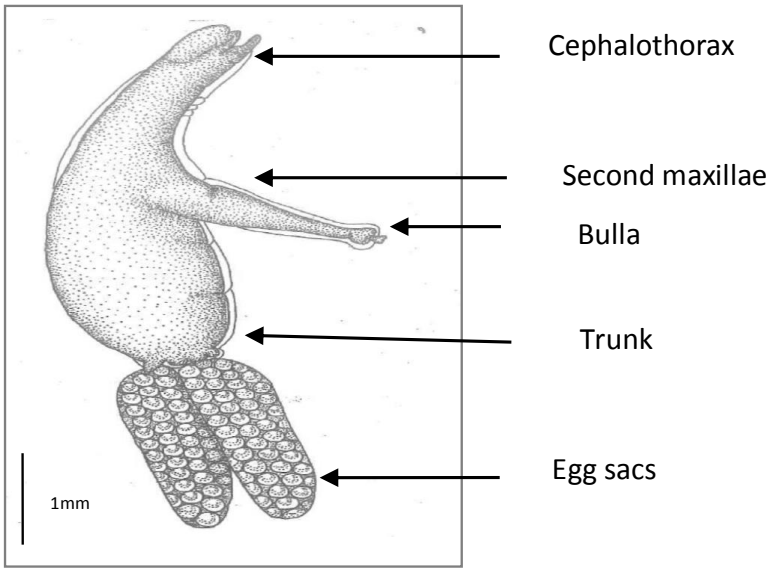

Figure 10. *Neobrachiella mugilis* (Kabata et al., 1971) According to Benmansour, 2001.

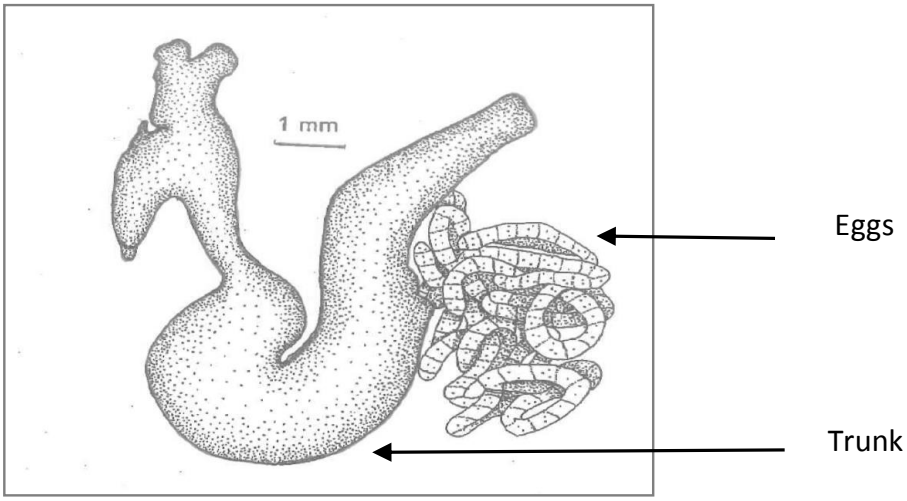

Figure 11. *Lernaeocera lusci* (Bassett-Smith, 1896) According to Benmansour, 2001.

of Chondrichthyan fish among them, 5 species of Rajidae (*Raja batis, Raja clavata, Raja laevis, Raja maevus* and *Raja radiata*) (Boxshall, 2001). In Tunisia, it was harvested for the first time on *Symphodus tinca* by Benmansour and Ben Hassine (1997).

The phenomenon of host specificity is the extent to which a parasite is restricted in the range of hosts it utilizes. Levels of host specificity can range through a continuum from high, with the parasite species occurring on only a single host species, to low, with the parasite species occurring on a wide range of phylogenetically unrelated host species (Boxshall, 1998).

The data concerning the dominance of stenoxenous species was different from the results recorded by Raibaut et al. (1998) and Benmansour and Ben Hassine (1997). Those authors showed that the large majority of copepod species are oioxenous. This difference between our results and those of the other authors is probably explained by the small taxonomical and geographical scale of our study. Sasal (1997) proved that studies conducted at different scales may lead to opposite conclusion.

Conclusion

Finally, it is very interesting to note that copepod species

Table 1. Fish species with their epidemiological characteristics, found to be infested by parasitic copepods.

Host	NEF	NIF	Copepods	P(%)	I	A	Location on the host	Specificity
Teleost fishes								
Boops boops	39	0	**********	****	****	****	*****	*****
Caranx rhonchus	40	0	**********	****	****	****	*****	*****
Spicara maena	42	0	**********	****	****	****	*****	*****
Merluccius merluccius	30	3	*Lernaeocera lusci*	10	1	0.10	Gills	O
		2	*Neobrachiella Merluccii*	6.66	1	0.06	Gills	O
Liza saliens	37	2	*Caligus pageti*	5.4	1	0.05	Fins	S
		4	*Neobrachiella mugilis*	10.8	1	0.10	Fins	S
Mullus barbatus	30	9	*Hatschekia mulli*	29.03	1	0.29	Gills	S
Mullus surmuletus	31	0	**********	****	****	****	*****	*****
Scomber scombrus	45	4	*Clavelissa scombri*	6.66	1.5	0.08	Gills	O
Sarpa salpa	31	3	*Clavellotis pagri*	9.67	1	0.09	Gills	S
Scorpaena porcus	30	0	**********	****	****	****	*****	*****
Scorpaena scrofa	30	0	**********	****	****	****	*****	*****
Sparus auratus	30	4	*Clavellotis fallax*	13.33	1	0.13	Gills	S
Chondrichthyan fishes								
Dasyatis centroura	38	0	**********	****	****	****	*****	*****
Pteromylaeus bovinus	48	0	**********	****	****	****	*****	*****
Raja clavata	52	3	*Caligus elongatus*	5.76	1	0.05	Gills	E
Rhinobatos cemiculus	32	0	**********	****	****	****	*****	*****
Scyliorhinus canicula	60	5	*Lernaeopoda galei*	8.33	1	0.08	Cloaca	E
Mustelus mustelus	60	3	*Lernaeopoda galei*	5	1	0.05	Cloaca	E
Mustelus punctalatus	49	1	*Lernaeopoda galei*	2.04	1	0.02	Cloaca	E

(NEF: Number of examined fishes; NIF: Number of infested fishes; P (%): Prevalence; I: mean intensity; A: Abundance; O: Oixenous; S: Stenoxenous, E: Euryxenous; *: absence of parasites).

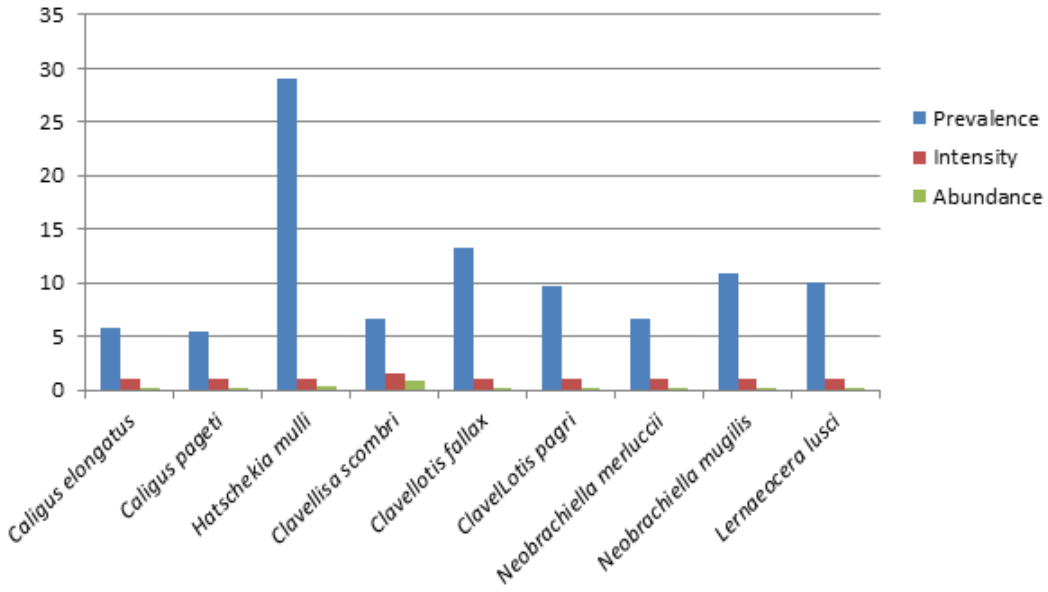

Figure 12. Epidemiological parameters of Copepods collected.

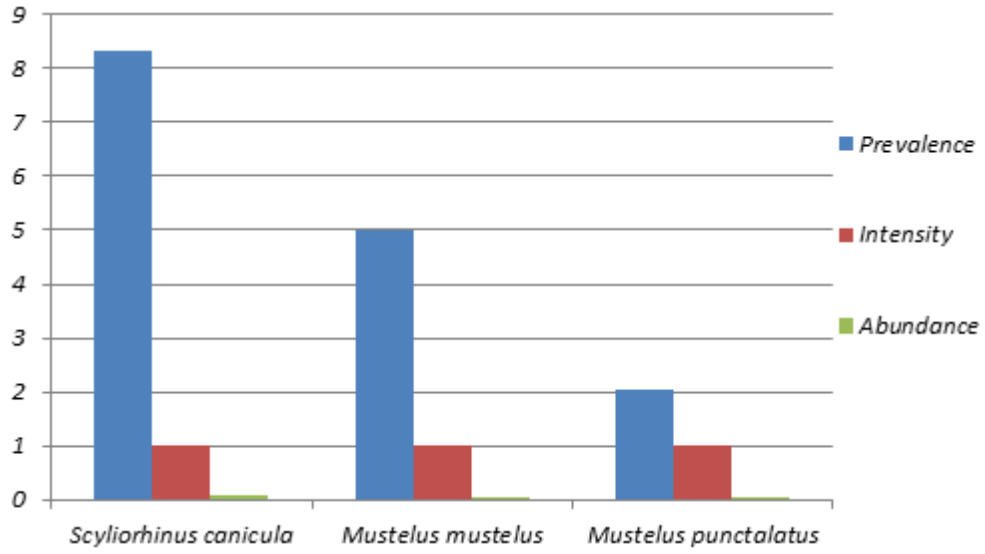

Figure 13. Epidemiological parameters of *Lernaeopoda galei*.

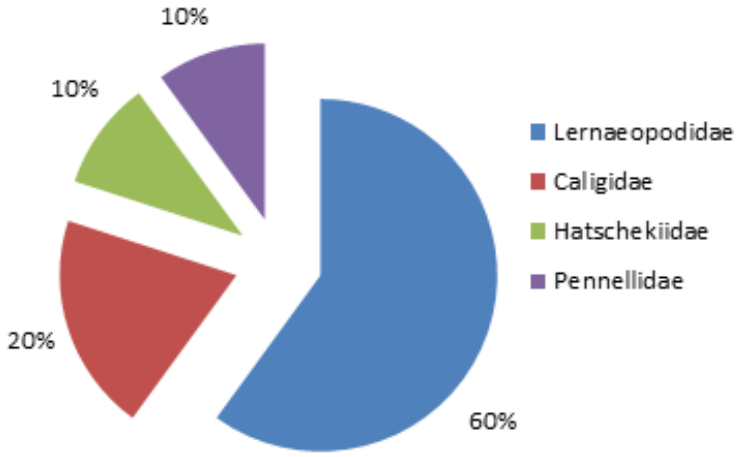

Figure 14. Richness of parasites per family.

can cause serious fish diseases, even though they present low parasitic indices. According to Company et al. (1999) and Athanassopoulou et al. (2001) parasites that have a low prevalence and abundance and minor pathological effects on their hosts in the wild can easily spreads in populations, confined to rearing systems and causing serious outbreaks of epizootic diseases.

Therefore, further investigations are still needed to deepen our knowledge of these ectoparasites of Teleost and Chondrichthyan fish.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Full Length Research Paper

Clinico-mycological profiles of dermatophytosis in Jaipur, India

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Dermatophytosis poses a serious crisis to the socio-economically backward population. The infections are caused by three anamorphic genera; *Epidermophyton, Trichophyton* and *Microsporum* which are distributed around the world. The objective of this study was to find out the occurrence, distribution and prevalence of dermatophytes causing human dermatomycosis in various categories of patients in Jaipur (Rajasthan), India. One hundred samples were collected, including infected skin and nails from Dermatology Department, Sawai Maan Singh Hospital, Jaipur for a period of June 2014 to January 2015. Before sample collection, the infected area was cleaned with 70% ethyl alcohol. Skin samples were collected with the help of sterilized scalpel and nail samples by clipping. Identification of causative pathogens was done by performing lacto-phenol cotton blue mount. Out of 100 samples, 79 were found positive by KOH examination and out of them 53 confirmed by culture. In the present study, *Trichophyton rubrum* (20.7%) was the predominant pathogen followed by *Trichophyton mentagrophytes* (16.9%) and *Trichophyton interdigitale* (13.2%). Tinea corporis was the most common clinical type reported in all age groups. The second most common clinical type was Tinea cruris. These infections were observed more frequently in the age group of 21 to 30 (55%) followed by 31 to 40 (24%).

Key words: Dermatophytosis, Tinea corporis, *Trichophyton, Microsporum, Epidermophyton*.

INTRODUCTION

Dermatophytosis constitutes an important public health problem, not only in developing countries, but also in immuno-compromised patients worldwide (Walsh and Groll, 1999; Ghannoum et al., 2003; Carrillo-Munoz et al., 2008). Dermatophytosis is superficial infection of keratinised tissue caused by an organism of three genera of fungi known as Dermato-Phyton (Bhadauria et al., 2001). The etiological agents of the dermatophytosis can be categorized into one of the three genera: *Microsporum, Epidermophyton* and *Trichophyton* (Ghannoum and Isham, 2009). They possess keratinophilic and keratinolytic properties (Simpanya, 2000).

The typical infections of dermatophytes are generally referred to as ringworm infections due to their ringlike outer shell. These infections are also recognized as 'Tinea infections' and are named according to the location of the lesions on the body e.g. Tinea cruris refers to ringworm infection of the groin area. Since these

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infections are frequently confused with additional skin disorders, it is, therefore, necessary to make an early laboratory diagnosis for better management of these conditions (Bhatia and Sharma, 2014; Huda et al., 1995). The warm and humid climate of India makes dermatophytosis a very common superficial fungal infection of skin (Niranjan et al., 2012).

Dermatophytes typically do not affect the mucous membranes, but slightly affect the keratinized tissues and extend by direct contact from infected human beings (anthropophilic organisms), soil (geophilic organisms) and animals (zoophilic organisms) by the indirect way from fomites. Although, the clinical symptoms of dermatophytosis may differ depending on the affected part of the body, itching is the most frequent symptom in humans (Nweze, 2010).

The lifestyle in societies, contact with animals and prolonged use of antibiotics, antineoplastic and corticosteroids drugs are some of the factors that contribute to the increase in the risk of infection by fungi, especially by dermatophytes (Rippon, 1985). Dermatophytosis, considered as zoonosis, have created more public health concerns due to close contact between humans and animals such as dogs, birds, cats, and small rodents or pocket pets. The clinical symptoms may not create a serious threat, but effective treatment is usually time-consuming and costly (Javed, 2015). The dermatomycosis takes place by contact of soil-to-human, animal-to-human and human-to-human spread. Recently, we frequently examine patients with widespread dermatomycosis on groin area and glabrous skin, on hands, on face, on the scalp and on foot.

The major objective of the study was to discover the prevalence, distribution and prevalence of dermatophytes causing human dermatomycosis in various categories of patients in Jaipur (Rajasthan), India.

MATERIALS AND METHODS

In the present study, sample collection was conducted for a period of 8 months from June 2014 to January 2015. The skin scrapings, nail clippings were collected from Dermatology Department, Sawai Man Singh Hospital in Jaipur district. A detailed clinical history was elicited from all the patients as per the performa. The following additional points were also recorded: name, gender, age of patient, body part involved, the presence of inflammatory margin, symptoms, duration of illness.

Sample collection

A total 100 skin scrapings and nails were collected. However, a proper explanation of the study was addressed to the patients and the consent was taken from them before collection of the sample. The first step of the sample collection was cleaned of the infected area with 70% ethyl alcohol and ensured that it was totally dry. The skin sample was scraped with the help of a sterilized scalpel from a peripheral area of the lesion and nail sample by clipping. Sample materials were transported in dry, strong black paper folded in the manner of an herbarium packet, and transferred to the laboratory as soon as possible for direct microscopic examination and culturing (Weitzman and Summerbell, 1995; Kane and Summerbell, 1997).

KOH mount

The skin scrapings were treated with an aqueous solution of 10% potassium hydroxide (KOH) and 20% for nail clipping and gently heat, examined after 5 min under the microscope for the presence of fungal hyphae (Rebell and Taplin, 1970). Heat may help to increase the lytic activity of KOH (Behzadi and Behzadi, 2003; Hainer, 2003; Webster and Weber, 2007; Deacon, 2009; Garg et al., 2009; Behzadi and Behzadi, 2012; Moriarty et al., 2012).

Culture and maintenance

Skin scrapings were inoculated on Sabouraud's dextrose agar with Chloramphenicol and Cycloheximide (Himedia) by slant method. The inoculated slants were placed in a mycological incubator at 26 ± 2°C for 14 to 21 days. After the isolation, dermatophytic fungi were again subcultures on SDA slants for purifications. The purified dermatophytic fungi were maintained and preserved at 4°C for further future analysis. Isolates of dermatophytes were identified by examining macroscopic and microscopic characteristics of their colony. Rate of growth, texture, topography, and pigmentation of the front and the reverse side of the culture were employed for the macroscopic identification.

Microscopic identification of dermatophytes

Isolates were examined microscopically by removing a portion of aerial mycelium with an inoculating needle. The material was placed on a glass slide in a drop of Lacto phenol cotton blue and the matted mycelium was gently removed by coverslip. A cover-slip was then placed on the side and excess of stain removed with blotting paper. The morphology was then observed under microscope. The identification was based on features such as the organization of hyphae (spiral, pencil shaped, pyriform, septations, etc.), microconidia and macroconidia (drop like, tear shaped, in bunches, spherical, abundance or rare, etc.) (Bhatia and Sharma, 2014).

RESULTS

In the present study, out of 100 samples, 79 (79%) were KOH positive and out of them 53 (67%) cultures positive.

The data presented in Table 1 shows the occurrence of various clinical types and etiological agents of ringworm infections. *Trichophyton rubrum* was the major etiological agent reported from 11 cases (20.7%). It was isolated from Tinea corporis and T. cruris clinical types. The second etiological agent *Trichophyton mentagrophytes* (16.9%) followed by *Trichophyton interdigitale* (13.2%),

Table 1. Distribution of fungal isolates from different cases.

S/N	Clinical types	Tinea corporis	Tinea cruris	Tinea corporis + cruris	Tinea pedis	Tinea manuum	Tinea faciei	*Onychomycosis*	Tinea capitis	Tinea barbae	Total Cases	%
1	No. of cases examined	42	38	8	4	3	1	1	2	1	100	-
2	No. of cases positive by microscopy	30	32	8	2	3	1	1	1	1	79	79
3	No. of cases positive by cultures	20	20	6	2	1	1	1	1	1	53	53
4	No. of cases negative by microscopy	12	6	0	2	0	0	0	1	0	21	21
	Species isolated											
a	*T. rubrum* (11)	5	6	-	-	-	-	-	-	-	11	20.7
b	*T.mentagrophytes* (9)	1	7	1	-	-	-	-	-	-	9	16.9
c	*T. interdigitale* (7)	3	3	-	1	-	-	-	-	-	7	13.2
d	*T.verrucosum* (5)	1	2	2	-	-	-	-	-	-	5	9.4
e	*M.gypseum* (3)	-	2	1	-	-	-	-	-	-	3	5.6
f	*T. equinum* (2)	-	-	-	-	-	-	1	1	-	2	3.7
g	*T. erinacei* (2)	-	-	-	-	1	1	-	-	-	2	3.7
h	*M.nannum* (2)	2	-	-	-	-	-	-	-	-	2	3.7
i	*T. terrestre* (1)	1	-	-	-	-	-	-	-	-	1	1.8
	Other associated fungi											
j	*Emericiella* (4)	2	-	1	1	-	-	-	-	-	4	7.5
k	*Histoplasma capsulatum* (1)	-	-	-	-	-	-	-	-	1	1	1.8
l	*Chrysosporium indicum* (1)	1	-	-	-	-	-	-	-	-	1	1.8
m	*Chrysosporium queenslandicum* (1)	1	-	-	-	-	-	-	-	-	1	1.8
n	*Fusarium oxysporum*(1)	1	-	-	-	-	-	-	-	-	1	1.8
o	*Fusarium equiseti* (1)	1	-	-	-	-	-	-	-	-	1	1.8
p	*Fusarium solani* (1)	1	-	-	-	-	-	-	-	-	1	1.8
q	*Aspergillus niger* (1)	-	-	1	-	-	-	-	-	-	1	1.8
	Total No.	20	20	6	2	1	1	1	1	1	53	100
	Percentage (%)	37.7	37.7	11.3	3.7	1.8	1.8	1.8	1.8	1.8	100	

Trichophyton verrucosum (9.4%), *Microsporum gypseum* (5.6%), *Trichophyton equinum* (3.7%), *Trichophyton erinacei* (3.7%), *Microsporum nanum* (3.7%), and *Trichophyton terrestre* (1.8%).

Some associated fungi were also isolated and Identified from *Tinea* patients such as *Emericiella, Histoplasma capsulatum, Chrysosporium indicum, Chrysosporium queenslandicum, Fusarium oxysporum, Fusarium equiseti, Fusarium solani* and *Aspergillus niger*.

In the present study, T. corporis was found to be the most common disease 42:100%. T. cruris was

Table 2. Clinical analysis of superficial mycoses in various age groups.

Clinical type	11-20 years	21-30 years	31-40 years	41-50 years	Total
Tinea cruris	4	22	8	4	38
Tinea corporis	4	22	12	4	42
Tinea cruris+corporis	0	5	3	0	8
Tinea pedis	0	2	1	1	4
Tinea faciei	0	0	0	1	1
Tinea manuum	0	2	0	1	3
Onychomycosis	0	0	0	1	01
Tinea capitis	1	1	0	0	02
Tinea barbae	0	1	0	0	1
Total	9	55	24	12	100

Table 3. Skin scrapings collected from various types of *Tinea* infection from SMS Hospital.

Types of employment	Tinea corporis	Tinea cruris	Tinea corporis +cruris	Tinea pedis	Tinea faciei	Tinea manuum	Tinea capitis	*Onychomycosis*	Tinea barbae	Total
Labour class	9	12	2	2	-	1	-	-	-	26
Private employee	12	10	1	-	-	1	-	-	1	25
Student	11	10	1	1	-	-	1	-	-	24
Self employed	6	5	1	-	-	-	-	-	-	12
Farmer	4	1	2	1	1	1	-	1	-	11
Business class	-	-	1	-	-	-	1	-	-	02
Total	42	38	08	04	01	03	02	01	01	100

the second clinical type reported in 38:100%, followed by Tinea corporis + T. cruris 8:100%, Tinea pedis 4:100%, Tinea manuum 3:100%, Tinea capitis 2:100%, Tinea faciei 1:100%, Tinea *barbae* 1:100% and *onychomycosis* 1:100% (Table1).

Table 2 represents that Tinea infection was the most common in the age group of 21 to 30 years followed by 31 to 40, 41 to 50 and 11 to 20 years. T. corporis was the most common clinical type of infection in age group of 21 to 30 and 31 to 40 years.

Table 3 represents the prevalence of various clinical types of ringworm infection in different categories. In all clinical types of Tinea infection, patients were commonly reported from the labour class as they work in unhygienic environment and poor socioeconomic background compared to other patients. On the other hand, T. corporis was common in private employee (12%) followed by student patients (11%).

DISCUSSION

The occurrence of dermatophytosis has increased globally in recent years, especially in immunocom-

promised patients (Borman et al., 2007). Few studies have investigated the etiology of superficial fungal infections in the developing world, and accordingly, there is less knowledge of any changes to their epidemiology (Saunte et al., 2008; Hasan et al., 2011).

The present study highlights the prevalence of different dermatophytic species concerned in different Tinea infections in Jaipur. The common occurrence of this species in Jaipur may be due to hot and dry climate in summer. The temperature exceeds 46°C with high humidity during the monsoon season (rainy season) which is the favorable condition for the occurrence of superficial mycoses. Various studies have been conducted to discover the occurence of dermatophytosis in different parts of the country including 65% in Chandigarh (Chakrabarti et al., 1992), 70.5% in West Bengal (Grover and Roy, 2003), 52.78% in Gujarat (Bhavsar et al., 2012), 78.9% in Chennai (Venkatesan et al., 2007), 61.56% in Andhra Pradesh (Maruthi et al., 2012), 85.5% in Madhya Pradesh (Pandey and Pandey, 2013), 36.6% in Himachal Pradesh (Bhatia and Sharma, 2014), 86% in Uttar Pradesh (Kumar et al., 2014), 78.53% in Karnataka (Reddy et al., 2012), 81.36% in Rajasthan (Jain et al., 2014), 57.89% in Manipur (Singh et al., 2015) and few other states.

In the present study, *Trichophyton rubrum* (20.7%) was the most prevalent dermatophytic species in jaipur. Bhadauria et al. (2001) reported 34% occurrence of *T. rubrum* in Jaipur area during 1999 to 2001. Jain et al. (2014) reported *T. rubrum* (32.1%) was the most major dermatophytic species in Jaipur. The second most common etiological agent of dermatophytosis was *Trichophyton mentagrophytes* (16.9%). This result correlated with the results of Jain et al. (2014). They reported 14.3% occurrence of *T. mentagrophytes* in their study. Kumar et al. (2014) also reported *T. mentagrophytes* (17.9%) was the second etiological cause of dermatophytosis in his research work.

In the present study, the maximum occurrence of Tinea infection was observed in 21 to 30 years age group followed by 31 to 40 year age group. The earlier researchers reported the maximum occurrence of dermatophytosis in 21 to 30 age group (Patwardhan et al., 1999; Jain et al., 2014; Goyal et al., 2015).

In the present study, T. corporis (42%) was the most common clinical type followed by T. cruris. Bhadauria et al. (2001) reported 60% prevalence of T. corporis. Jain et al. (2014) also reported most common prevalence of T. corporis followed by T. cruris in his study. The second clinical type of dermatophytosis in the present study was T. cruris (38%). In the earlier report by Patwardhan et al. (1999), T. cruris was the second most common clinical type in his study. Venkatesan et al. (2007) and Jain et al. (2014) reported that T. cruris was the second common clinical type of dermatophytosis. *T. rubrum* was the most common etiological agent in T. corporis and T. cruris followed by *T. mentagrophytes*.

Conclusion

The present research concluded that the climatic conditions of Jaipur are favourable for dermatophytosis in the population. The present research work also highlights that T. corporis was the major clinical type infection followed by T. cruris. *T. rubrum* was the predominant species followed by *T. mentagrophytes* and *Trichophyton interdigitale.* Unhygienic conditions with poor socio-economic class, frequent migration of laborers, workers, regular visits of tourists in Jaipur district of Rajasthan may be some of the contributing epidermiological factors.

Conflict of interest

The authors have not declared any conflict of interest.

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