ORIGINAL ARTICLE

PRESUMPTIVE IDENTIFICATION OF CANDIDA SPECIES BY USING CHROMOGENIC AGAR IN COMPARISON WITH YEAST IDENTIFICATION PROTOCOLS IN A TERTIARY CARE HOSPITAL

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HOW TO CITE THIS ARTICLE:
Anjana Gopi, Neetha S. Murthy. "Presumptive Identification of Candida Species by using Chromogenic Agar in Comparison with Yeast Identification Protocols in a Tertiary Care Hospital". Journal of Evidence based Medicine and Healthcare; Volume 1, Issue 13, December 01, 2014; Page: 1604-1613.

ABSTRACT: Background and Objective: Candida is yeast like fungus and is the most common fungal pathogen causing disease in human beings. Although Candida albicans remains the most common cause of human Candidiasis, the frequency of infection attributed to other members of the genus is also increasing.[1,2] Speciation of candida can be done using CHROM agar, which is a differential culture medium and facilitates the isolation and identification of some clinically important species. The objective of this study was to isolate and identify Candida from various clinical samples using standard yeast identification protocols and rapid identification methods. To study the antifungal susceptibility pattern associated with the isolates thus obtained and to bring out the various risk factors associated with candidiasis. **METHOD:** Positive samples for candidiasis were collected from 250 patients in our institute from August 2013 to February 2014. Standard yeast identification protocols, CHROM agar media and Rapid Hicandida™ Identification Kits were used for speciation. Antifungal susceptibility testing was done by Modified Kirby Bauer Disc diffusion technique on Muller Hinton agar with 2% glucose and Methylene blue as per CLSI guidelines to detect the sensitivity to amphotericin B, fluconazole, and voriconazole. **RESULTS:** Among the 250 culture positive cases, 118 (47.2%) C.albicans, 64 (25.6%) C.tropicalis, 27 (10.8%) C.dubliniensis, 25 (10%) C. glabrata, 16 (6.4%) C. parapsilosis were obtained. The antifungal susceptibility pattern suggested 14% of the isolates were resistant to fluconazole, 12% to voriconazole and 1.2% isolates were resistant to amphotericin B. **CONCLUSION:** Among the 250 isolates, non-Candida albicans (NCA) species were 132 (52.8%). Candida tropicalis was predominant among the non-Candida albicans isolates. The antifungal susceptibility pattern suggested that a major portion (62.85%) of the fluconazole resistant isolates were NCA species. The risk factors for candidiasis noted in this study were diabetes mellitus, antibiotic therapy, low birth weight and pregnancy.

KEYWORDS: Candida speciation, Non-Candida albicans, CHROM agar, Rapid identification kit, Antifungal susceptibility testing.

INTRODUCTION: Infections due to Candida species have increased in the past three decades due to the use of potent antibacterial agents, immunosuppressive and cytotoxic drugs.[3] In a 7-year long study analyzing nosocomial Blood stream infection (BSI) in hospitals in the United States (Surveillance and Control of Pathogens of Epidemiological Importance [SCOPE]), Candida species was found to be the fourth most common cause of BSI in a hospital setup.[4]

Invasive candidiasis is associated with substantial morbidity and mortality and is difficult to diagnose due to lack of specific signs and symptoms.[5] Identification of Candida to species

level is definitely warranted as there is increase in the incidence of non-Candida albicans infections.[3] Since molecular techniques are very expensive, usage of CHROM agar and Rapid Identification kit for species identification would be of benefit for easy and rapid speciation.[5]

Although Candida albicans remains the most common cause of human Candidiasis, the frequency of infection attributed to other members of the genus is also increasing. This is primarily due to the increase in the number of at risk individuals, particularly those with impaired immunity, such as transplant recipients, cancer patients receiving chemotherapy, and human immunodeficiency virus-infected patients.[6]

MATERIAL AND METHODS: A prospective study was undertaken at our institute for a period of 7 months from August 2013 to February 2014. Candida speciation was carried out in the following manner. Clinical specimens received in the laboratory were cultured on two tubes of Sabouraud dextrose agar (SDA) with and without actidione respectively and incubated at 37°c.

White to cream-colored, pasty and smooth colonies appeared in 3-4 days. These colonies morphologically resembling the members of genus candida were subjected to gram staining. On microscopic examination they appeared as gram-positive budding yeast like cells. (Figure-1/ table-1).

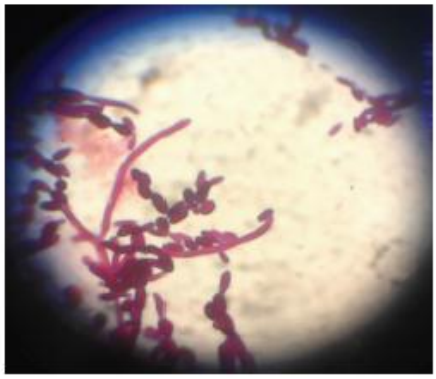


Figure-1/table-1 gram positive budding yeast like cells with pseudohyphae.

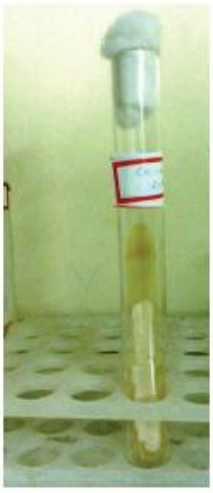


Figure-2/table-2 growth on SDA

Subsequently the Germ tube test was done and the isolates were classified as Germ tube test positive and negative. The germ tube positive samples were further incubated at 45°c.

The isolates were then inoculated on CHROM agar (Hi media) from the SDA slopes. (Figure-3/ table-3) CHROM agar is a novel, differential culture medium that is claimed to facilitate the isolation by colorimetric presumptive identification.[7,8] (Figure-4/table-4) Candida species and the corresponding color produced on CHROM agar (Figure-4/table-4)

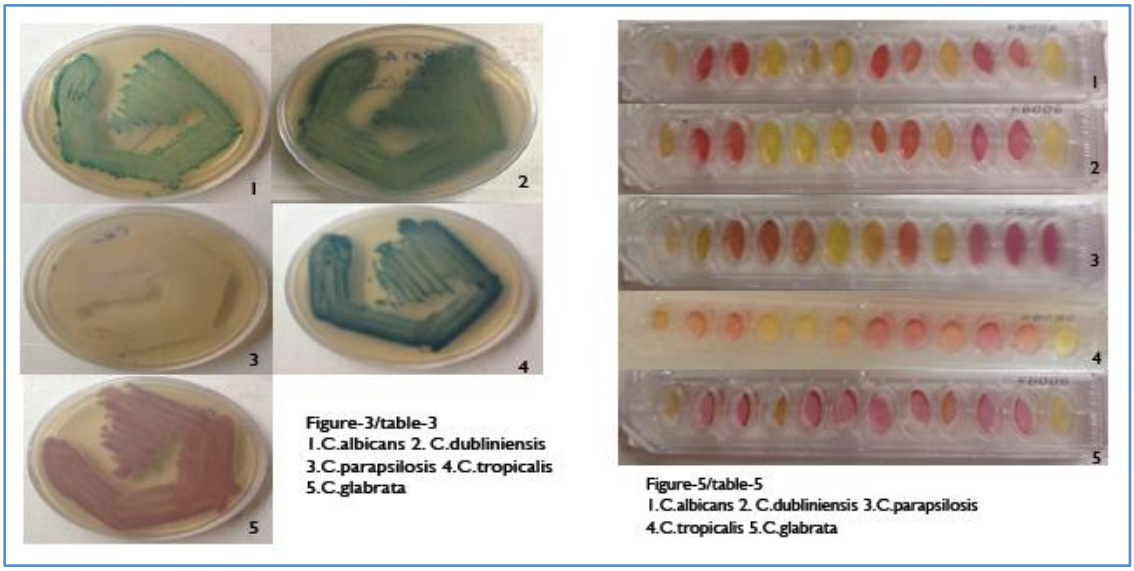


Figure-3/table-3
1.C.albicans 2. C.dubliniensis
3.C.parapsilosis 4.C.tropicalis
5.C.glabrata

Figure-5/table-5
1.C.albicans 2. C.dubliniensis 3.C.parapsilosis
4.C.tropicalis 5.C.glabrata

C. albicans	Light green
C. dubliniensis	Dark green
C. parapsilosis	White
C. tropicalis	Dark blue
C. glabrata	Pink

Rapid Identification kit KB006 (Hi media India) was used for identification and differentiation of candida species. It is a standardized colorimetric identification system utilizing twelve conventional biochemical tests. The test is based on the principle of pH changes and substrate utilization indicated by change in color of media. Results of the rapid identification kit using 12 standard biochemical tests (Figure-5/table-5) were interpreted as per the manufacturer's instructions and were found to be in coordination with the CHROM agar identification.

Figure-5/table-5 Result Interpretation

Well number	Test	Principle	Original colour of medium	Positive reaction	Negative reaction
1	Urease	Detects	Orangish	Pink	Orangish
		Urease	yellow		yellow
		enzyme			
2	Melibiose	Melibiose	Pinkish Red	Yellow	Red/Pink
		utilization	/ Red		
3	Lactose	Lactose	Pinkish Red	Yellow	Red/Pink
		utilization	/ Red		

4	Maltose	Maltose	Pinkish Red	Yellow	Red/Pink
		utilization	/ Red		
5	Sucrose	Sucrose	Pinkish Red	Yellow	Red/Pink
		utilization	/ Red		
6	Galactose	Galactose	Pinkish Red	Yellow	Red/Pink
		utilization	/ Red		
7	Cellobiose	Cellobiose	Pinkish Red	Yellow	Red/Pink
		utilization	/ Red		
8	Inositol	Inositol	Pinkish Red	Yellow	Red/Pink
		utilization	/ Red		
9	Xylose	Xylose	Pinkish Red	Yellow	Red/Pink
		utilization	/ Red		
10	Dulcitol	Dulcitol	Pinkish Red	Yellow	Red/Pink
		utilization	/ Red		
11	Raffinose	Raffinose	Pinkish Red	Yellow	Red/Pink
		utilization	/ Red		
12	Trehalose	Trehalose	Pinkish Red	Yellow	Red/Pink
		utilization	/ Red		

Test	Urease	Melibiose	Lactose	Maltose	Sucrose	Galactose	Cellobiose	Inositol	Xylose	Dulcitol	Raffinose	Trehalose
C.albicans	-	-	-	+	+	+	-	-	+	-	-	+
C.dubliniensis	-	-	-	+	+	+	-	-	+ *	-	-	+
C.parapsilosis	-	-	-	+	+	-	-	-	+	-	-	-
C.tropicalis	-	-	-	+	+	+ *	+	-	+	-	-	+
C.glabrata	-	-	-	+	-	-	-	-	-	-	-	+

Note: Based on % strains showing reactions following symbols have been assigned from laboratory results and standard references.

\+ * Strain variation

\+ Positive reaction (more than 90 %) - Negative reaction Not detected!

The antifungal susceptibility testing was carried out by disk diffusion method on using Mueller-hinton agar with 2% glucose and 0.5 µg/ml Methylene blue for Fluconazole (10mcg), Voriconazole(1mcg), Amphotericin(10mcg) as per Approved Standard M44-A2. Each inoculum was standardized to 0.5 Mc Farland units and the zone break points were interpreted as per the CLSI guidelines.[9] (Figure-6/table-6)

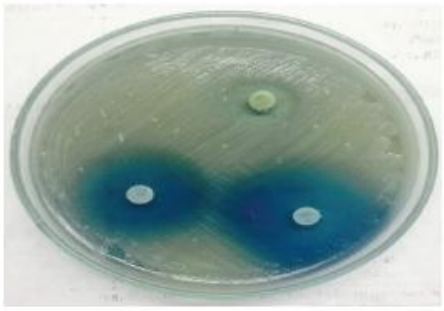
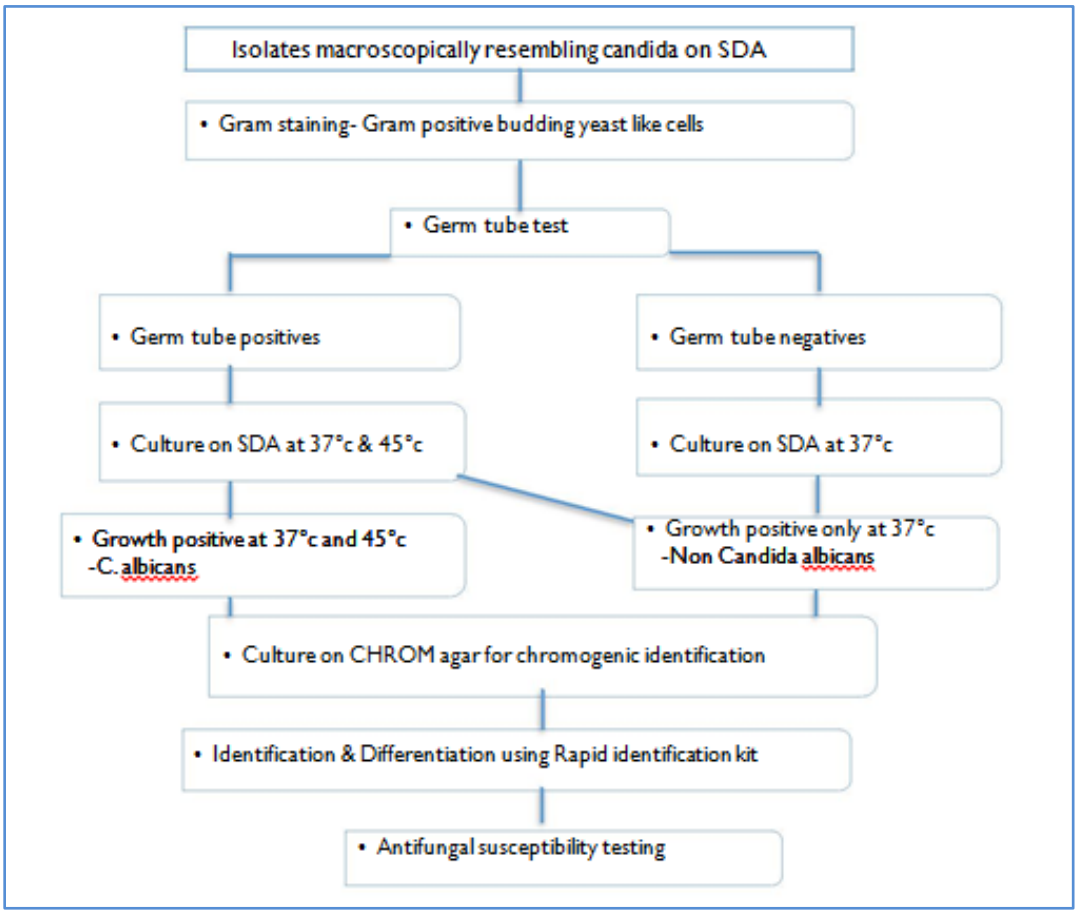
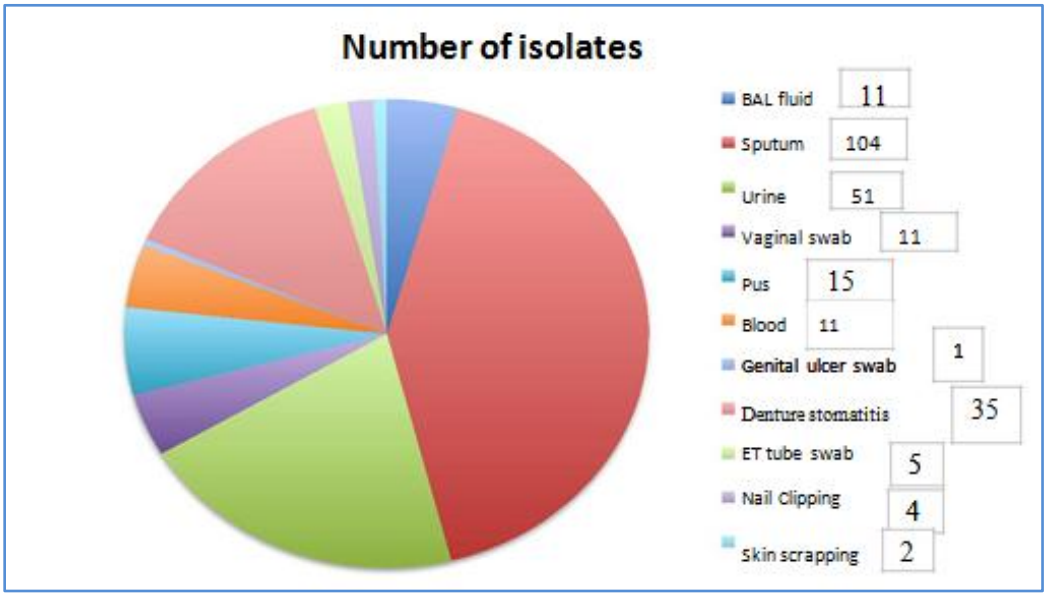


Figure-6/table 6 - antibiotic sensitivity testing with fluconazole, voriconazole and amphotericin.



RESULTS: 250 clinical isolates of candida species confirmed by standard yeast identification protocols were included in the study. Out of the 250 isolates it was found that 128 isolates (51.2%) were from males and 122(48.8%) from female patients. The split up of the isolates has been portrayed in figure-7/table-7.

Figure-7/table-7:



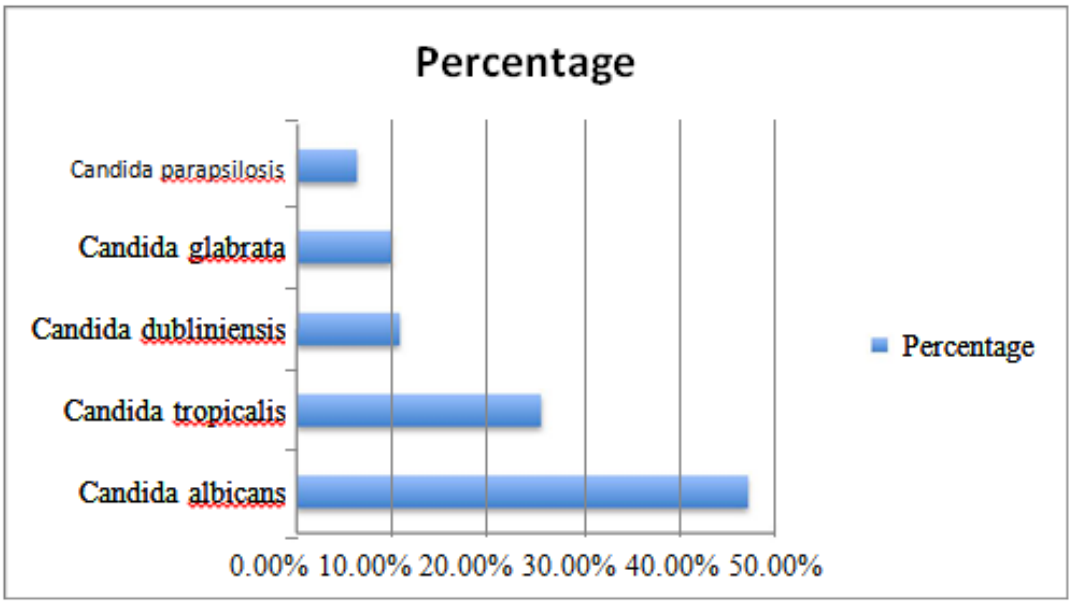
The age-wise distribution of candidiasis was as follows. Candidiasis appears to be maximum between the ages of 51 to 60 years and minimum between the ages of 11-20 years. [Figure-8/table-8]

Figure-8/table-8 Age distribution

Age distribution	Number of cases
0-10	13
11-20	4
21-30	5
31-40	31
41-50	33
51-60	93
>60	71

Candida albicans constituted 47.2% of the isolates and Non candida albicans accounted for 52.8% of the isolates. Among the non albicans species Candida tropicalis [25.6%] was the highest isolated species. [Figure-9/table-9]

Figure-9/table-9: Species isolation



Most of the isolates of candida albicans and NCA were distributed between the age group 50-60 years. The Vaginal samples with candidiasis were most commonly isolated from women between the age groups of 20-40. (Figure-10/table-10)

Figure-10/table-10: Age wise species distribution

Species	<28 days	28-365 days	1-10 yrs	11-20 yrs	21-30 yrs	31-40 yrs	41-50 yrs	51-60 yrs	>60 yrs
C. albicans	1	-	4	-	12	10	17	42	32
C. tropicalis	-	-	-	3	4	5	8	29	15
C. parapsilosis	2	-	-	1	-	-	10	3	-
C. dubliniensis	-	-	-	-	-	13	-	12	2
C. glabrata	3	2	1	-	2	-	-	8	9

Diabetes mellitus was the most commonly associated risk factor, followed by the use of broad-spectrum antibiotics. Pregnancy and the use of steroids were the other risk factors recorded by our study. [Figure-11/table-11]

Figure-11/table-11 associated risk factors

Risk factors	Number= n/250	Percentage
Diabetes mellitus	94	37.6%
Broad spectrum antibiotics	42	16.8%
Pregnancy	36	14.4%
Steroids	32	12.8%
No identifiable risk factor	46	18.4%

The antifungal susceptibility of the isolates was as follows- Only two isolates of C.albicans and one isolate of C.tropicalis were found to be resistant to amphotericin. Whereas 14% isolates were resistant to fluconazole of which 62.8% were NCA isolates. (Figure-12/table-12)

Figure-12/table-12

	Fluconazole (10mcg)			Voriconazole (1mcg)			Amphotericin (10mcg)		
	S	I	R	S	I	R	S	I	R
C. albicans	103	2	13	105	1	12	116	-	2
C. parapsilosis	12	1	3	15	-	1	15	1	-
C. glabrata	16	2	7	15	2	8	23	2	-
C. tropicalis	57	1	6	59	-	5	63	-	1
Percentage	83.6%	2.4%	14%	86.8%	1.2%	12%	97.6%	1.2%	1.2%

DISCUSSION: The epidemiology of Candida infections has changed over the last two decades: The number of patients suffering from such infections has increased dramatically.

In the present study 52.8% of the isolates were found to be NCA, which is in coordination with other studies done by workers in India. As per the study done by Vijaya et al, 54.1% NCA isolates were obtained.[8] The predominant NCA isolated in our centre was C.tropicalis, which was similar in studies done by Ragini et al,[5] Vijaya et al,[8] and Saroj et al.[3]

The male to female ratio was found to be 1: 0.95 as per the study. Many studies including one done in Ahmedabad also showed male preponderance.[5][9]

According to the present study the maximum cases were isolated from patients belonging to age group above 50 years. However the vaginal swab isolates were maximum in the age group 21-40 years in co-ordination with the other studies conducted.[10]

The potential clinical importance of species-level identification of Candida species lies in the fact that they differ in the expression of virulence factors and antifungal susceptibility. For differentiation between different species of candida conventionally Germ tube test, chlamydospore formation, sugar fermentation and assimilation tests are being used which are laborious and time consuming. CHROM agar is a rapid method to differentiate between different

candida species. It facilitates identification of candida species in 24-48 hours. As per our study, CHROM agar and rapid identification kit were found to have the advantage of being technically simple and rapid. However Chrom agar was found to be more cost effective as compared to the rapid identification kit.

As per our study Diabetes was the most commonly associated risk factor followed by the use of antibiotics. It was found that 14% of the isolates were resistant to fluconazole with 62.8% being NCA isolates thereby showing an increased prevalence of fluconazole resistance in NCA isolates.

Studies in India by Kothari et al, Kumar et al, Xess et al, Adhikary et al-have also shown an increasing trend of fluconazole resistance.[11] Fluconazole and other triazoles have less activity against C.glabrata and other NCA isolates.[11]

Most of the isolates were sensitive to amphotericin but since the drug is highly nephrotoxic hence is not the first choice of treatment.[11] However lipid based formulations with superior side effect profiles are presently available for clinical use.

Further studies are required to assess the sensitivity to Echinocandins in Indian settings. The in vitro susceptibility testing of antifungal agents is becoming increasingly important because of the introduction of new antifungal agents and the recovery of clinical isolates that exhibit inherent or developed resistance to the available antifungal agents.

CONCLUSION: This study emphasizes the requirement of rapid and precise identification of Candida isolates to species level. CHROM agar provides for the rapid identification of candida in a resource-limited setting which is required for effective management strategies.

Effective treatment requires both early diagnosis and prompt initiation of therapy against fungal infection. The antifungal susceptibility testing will also enable clinicians to choose appropriate antifungal agents, thus decreasing patients' morbidity and mortality.

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Date of Submission: 11/11/2014.
Date of Peer Review: 12/11/2014.
Date of Acceptance: 17/11/2014.
Date of Publishing: 26/11/2014.