Anchor University Journal of Science and Technology (AUJST)
A publication of the Faculty of Science and Science Education, Anchor University Lagos
URL: journal.aul.edu.ng
Vol. 1 No 1, June 2020, Pp. 10 - 15

Antibiotic Susceptibility Profile of *Cronobacter sakazakii*

Oloninefa S. D.*, Daniyan, S. Y. , Abalaka, M. E.

Department of Microbiology, Federal University of Technology, Minna, Niger State, Nigeria

***Corresponding author:**
E-mail: : dami4nefa2013@gmail.com;
Phone: +2348038893341

Submitted 5 April, 2020
Accepted 17 May, 2020

Competing Interests: The authors declare no competing interests.

ABSTRACT

Background: *Cronobacter sakazakii* is an emerging opportunistic bacterium whose presence in powdered infant formulas has been reported in several literatures. Infections such as necrotizing enterocolitis, sepsis and severe meningitis common to both premature and full-term infants have been linked to *C. sakazakii*.
Objectives: The objectives were to determine antibiotic susceptibility profile of *C. sakazakii* using known antibiotics and to determine the time-kill of *C. sakazakii* by known antibiotics.
Methods: The antibiotics susceptibility of *C. sakazakii* and the time-kill were carried out using standard methods.
Results: The antibiotics susceptibility results showed that out of forty four *C. sakazakii* isolates tested, 44 (100%) were sensitive to Ciprofloxacin, 27 (61.36%) to Gentamycin and 17 (38.64%) to Streptomycin. The time-kill curve showed that both Ciprofloxacin and Gentamycin were able to kill *C. sakazakii* at 240 minutes.
Conclusion: Out of the three antibiotics effective against *C. sakazakii*, Ciprofloxacin and Gentamycin were able to kill *C. sakazakii* at 240 minutes. Thus, this suggests that Ciprofloxacin and Gentamycin will be effective therapeutic agents against *C. Sakazakii*.

Keywords: *Cronobacter sakazakii*, powdered infant formulas, antibiotic susceptibility profile, time- kill

INTRODUCTION

Cronobacter Sakazakii (previously known as *Enterobacter sakazakii*) is an opportunistic bacterium that survives and persists in dry and low-moisture environments such as powdered infant formula. Aigbekaen and Oshoma (2010) reported the presence of *C. sakazakii* from powdered foods locally consumed in Nigeria. The diseases caused by *C. sakazakii* affect all age groups but it is more pronounced in premature infants and those below two months (Henry and Fouladkhah, 2019). Life-threatening health complications (such as seizures, urinary tract infection, neonatal meningitis and sepsis) emanated from the infections caused by *C. sakazakii* (Hunter and Bean, 2013; CDC, 2015; Henry and Fouladkhah, 2019). In the same vein, Ezeh *et al*. (2018) and Feeney *et al*. (2014) reported that life -threatening infections which could lead to death in immunocompromised adults are also linked to *C. sakazakii*. Henry and Fouladkhah (2019) opined that environments such as manufacturing facilities where powdered infant formula (PIF) are produced, healthcare settings and domestic environments favoured the isolation of *C. sakazakii*.

Several outbreaks were recorded in neonates' intensive care as a result of infection caused by *C. sakazakii*. In May/ June 1994 in France, CDC reported that, thirteen neonates were infected, three died, June/ July 1998 in Belgium, twelve neonates developed necrotizing enterocolitis and two twin brothers died and a more serious one in Tennessee in 2001. These outbreaks have been linked to the contamination of PIF by *C. sakazakii* (CDC, 2001). In addition, two outbreaks recorded in New Zealand and France in 2004 was linked to two PIF. WHO (2004) reported the involvement of five hospitals in an outbreak of *C. sakazakii* infections. Out of the nine cases recorded in the outbreak in French, the dead of two infants were recorded. Among infants whose weights were less than 2 Kg, eight cases were recorded while one case was obtained in an infant born at 37 weeks whose weight was 3.25 Kg. On reviewing the practices in the hospitals, it was discovered that recommended hygienic practices to be maintained during the preparation, handling and storage of feeding bottles were not adhered to while storage of reconstituted formula beyond 24 h in domestic

refrigerators whose temperature cannot be controlled or traced was discovered in other four hospitals (WHO, 2004).

Furthermore, CDC (2001) reported that a total of five babies were lost in New Mexico due to *C. sakazakii* infection in 2008. This incident was of a great concern to the US Centres for Disease Prevention and Control on the consumption of PIF (CDC, 2001). More so, the feeding of two infants with infant formula made them to be infected with *C. sakazakii* in a hospital in Quertaro, Mexico in 2010. The two infants developed bloody diarrhoea. The first infant was treated with cefotaxime and vancomycin while the second was treated with clindamycin and amikacin and the two infants recovered (Jackson *et al*., 2015). As reported by Bowen *et al*. (2017), a baby girl who was healthy for twenty one days after a gestation period of twenty six weeks and a weight of 1405 g came down with sepsis in April, 2016. *C. sakazakii* was isolated from the cerebrospinal fluid and the blood samples. Although, she was treated with ampicillin and cefepime, she developed seizures and had liquefaction necrosis in the brain. PIF was not given to the infant but pasteurized human milk that was donated and maternal milk which was expressed were given to the infant in the first week after birth. *C. sakazakii* was isolated from the breast pump kit and the kitchen sink drain from the mother's home (Bowen *et al*., 2017).

Smirnova and Oktyabrsky (2018) reported in their work that bacterial susceptibility to antibiotics is in many cases strongly affected by their growth phase. The importance of an antibiotic to kill bacteria is very important in some clinical settings especially in the management of bacterial endocarditis or the treatment of patients with bacteremia in granulocytopenic (Fung-Tomc *et al*., 2000). This study therefore focuses on the antibiotic susceptibility profile of *C. sakazakii*.

MATERIALS AND METHODS

Collection of *Cronobacter sakazakii*

The isolates (*C. sakazakii*) used for the antibiotic susceptibility profile and time- kill were obtained from samples of powdered infant formulas subjected to microbial analysis for the isolation of *C. sakazakii* in the Microbiology Laboratory of Federal University of Technology, Minna, Nigeria.

Confirmation and Identification of *C. sakazakii*

C. sakazakii collected were confirmed by inoculating in buffer peptone water and enriched *Enterobacteriaceae* Enrichment Broth (EEB). The incubation was done at 37^{o}C for 24 h. Plates of HardyCHROM Sakazakii medium were inoculated with a loopful from EEB and incubated at 37^{o}C for 24-48 h in a dark incubator (Hardy Diagnostics Manual, 2011). The growth of *C. sakazakii* colonies (greenish colonies) on the HardyCHROM Sakazakii medium at the end of the incubation period were further identified by Gram Staining and biochemical tests as outlined by Brooks *et al*. (2007) and Cheesbrough (2010).

Standardization of *C. sakazakii*

The standardization of *C. sakazakii* was determined as described by McFarland (1907) and Murray *et al*. (2007).

Determination of Antibiotic Susceptibility Profile of *C. sakazakii*

Antibiotic susceptibility profile of *C. sakazakii* to known antibiotics was carried out according to Kirby-Bauer method (Willey *et al*., 2011). *C. sakazakii* was subjected to ten diffusion discs with antibacterial drugs. They comprised of Augmentin (30µg), Amoxicillin (25µg), Erythromycin (5µg), Tetracycline (10µg), Cloxacillin (5µg), Gentamycin (10µg), Cotrimoxazole (25µg), Chloramphenicol (30µg), Ciprofloxacin (10µg) and Streptomycin (30µg). The *C. sakazakii* was inoculated in Nutrient Broth (NB) and incubated for 24 h at 37^{o}C. *C. sakazakii* incubated for 24 h at 37^{o}C were streaked on Mueller-Hilton agar plates with the aid of sterile swabs. The plates were kept at the environmental temperature for a period of 5 minutes and then diffusion discs with antibacterial drugs were distributed on the plates and then incubated for 24 h at 37^{o}C. The results were interpreted by measuring zones of inhibition with the use of a millimetres scale rule. The results were presented as resistant or sensitive according to Clinical and Laboratory Standards Institute (2015).

Determination of the Time-Kill of Antibiotics Effective Against *C. sakazakii*

A standardized overnight culture was used to determine the time-kill of the antibiotics effective against *C. sakazakii*. One millilitre (1 ml) of the standardized inoculum was added to 9 ml Nutrient Broth containing the various antibiotics dilutions and 1 ml of the admixtures was withdrawn at various time intervals of 30, 60, 120, 180 and 240 minutes respectively. A dilution of tenfold (10^{-1}) was done and then plated on Nutrient Agar

in duplicates and incubated at 37°C for 24 h. The same procedure was repeated for the control without antibiotics. The population of *C. sakazakii* was enumerated and the values expressed in $\log_{10}$ cfu/ml after exposure to the antibiotics (Gengo *et al*., 1984).

RESULTS

Cultural Characteristics and Biochemical Tests

The colonies of *C. sakazakii* confirmed using HardyCHROM sakazakii medium appeared greenish. The Gram reaction showed that they were Gram negative while the biochemical tests revealed that they were all motile, catalase positive, methyl red negative, oxidase negative, positive for citrate utilization and nitrate reduction (Table 1).

Antibiotic Susceptibility Profile of *C. sakazakii*

Table 2 shows the results of antibiotic susceptibility profile of *C. sakazakii*. *C. sakazakii* were sensitive to Ciprofloxacin, Gentamycin and Streptomycin in the following order: 100%, 61.36% and 38.64%. The isolates were resistant to Chloramphenicol, Augmentin, Amoxicillin, Erythromycin, Tetracycline, Cloxacillin and Cotrimoxazole as they all had 0% each (Table 2)

Table 1: Cultural Characteristics and Biochemical Tests

	Morphology		Gram Reaction		Biochemical tests						
Isolate	**Colour**	**Shape**	**Reaction**	**Shape**	**Catalase**	**Citrate Utilization**	**Methyl red**	**Oxidase**	**Nitrate Reduction**	**Motility**	**Probable Organism**
n= 40	Red	Straight	-	Rods	+	+	-	-	+	+	*C. sakazakii*

n= Number of *C. sakazakii* tested

Table 2: Antibiotic Susceptibility Profile of *Cronobacter sakazakii*

	Antibiotics									
Number of Isolates	CIP	GEN	STR	CHL	AUG	AMO	ERY	TET	CLO	COT
3	3(100)	2(66.66)	1(33.33)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
1	1(100)	1(100)	1(100)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
1	1(100)	1(100)	1(100)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
2	2(100)	1(50)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
2	2(100)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
1	1(100)	1(100)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
2	2(100)	1(50)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
4	4(100)	3(75)	1(25)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
2	2(100)	1(50)	1(50)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
4	4(100)	2(50)	1(25)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
6	6(100)	5 (83.33)	2(33.33)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
3	3(100)	2(66.67)	1(33.33)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
5	5(100)	3(60)	2(40)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
3	3(100)	1(33.33)	2(66.67)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
5	5(100)	3(60)	4(80)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
44	44(100)	27(61.36)	17(38.64)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)

CIP-Ciprofloxacin; GEN-Gentamycin; STR-Streptomycin; CHL-Chloramphenicol; AUG-Augmentin; AMO -Amoxicillin; ERY-Erythromycin; TET-Tetracycline; CLO-Cloxacillin; COT-Cotrimoxazole

Time-Kill of Antibiotics Effective Against *C. sakazakii*

Figure 1 shows the results of the time-kill of effective antibiotics (Streptomycin, Ciprofloxacin and Gentamycin) and the control from 30-240 minutes expressed in log_{10}cfu/ml. The populations of the *C. sakazakii* were reduced in this order: Streptomycin ($3.5log_{10}$cfu/ml-$2.6log_{10}$cfu/ml), Ciprofloxacin ($3.3log_{10}$cfu/ml-$0.0log_{10}$cfu/ml) and Gentamycin ($3.0log_{10}$cfu/ml-$0.0log_{10}$cfu/ml). *C. sakazakii* was killed at 240 minutes by Ciprofloxacin and Gentamycin. However, the population of *C. sakazakii* increased from $3.7log_{10}$cfu/ml - $4.0log_{10}$cfu/ml when the control was used (Figure 1).

DISCUSSION

The *C. sakazakii* isolates were susceptible to Ciprofloxacin (100%), Gentamycin (61.36%) and Streptomycin (38.64%). This result agreed with the work of Aisha *et al*. (2013). The highest percentage recorded in Ciprofloxacin was similar to the results obtained in the study carried out by Jawad *et al*. (2013). However, the result was not in agreement with the work of Agbekaen and Oshoma (2010) in which Streptomycin was the most effective of all the antibiotics used during the susceptibility testing. The highest percentage of susceptibility recorded in Ciprofloxacin may be attributed to the initial population of the test organism, rate of diffusion of the antimicrobial agent and the rate of growth of the organism as opined by Hugo (1998). The susceptibility of the isolates to Gentamycin and Streptomycin (members of aminoglycosides) as revealed in Table 2 agreed with the work of Stock and Wiedemann (2002). More so, the isolates were resistant to Augmentin, Amoxicillin, Erythromycin, Tetracycline, Cloxacillin, Cotrimoxazole and Chloramphenicol as they all recorded 0% each (Table 2). This possibly may be due to insufficient antimicrobial agents and their rates of diffusion. The rate of growth of the organism may also be responsible for the resistance. *C. Sakazakii* resistance to Amoxicillin obtained in the study carried out by Agbekaen and Oshoma (2010) agreed with this study. .Similarly, the resistance of *C. Sakazakii* to Amoxicillin and Tetracycline agreed with the works of Mohammeddaman *et al*. (2014). Furthermore, the kill-time curve showed reductions in the population of

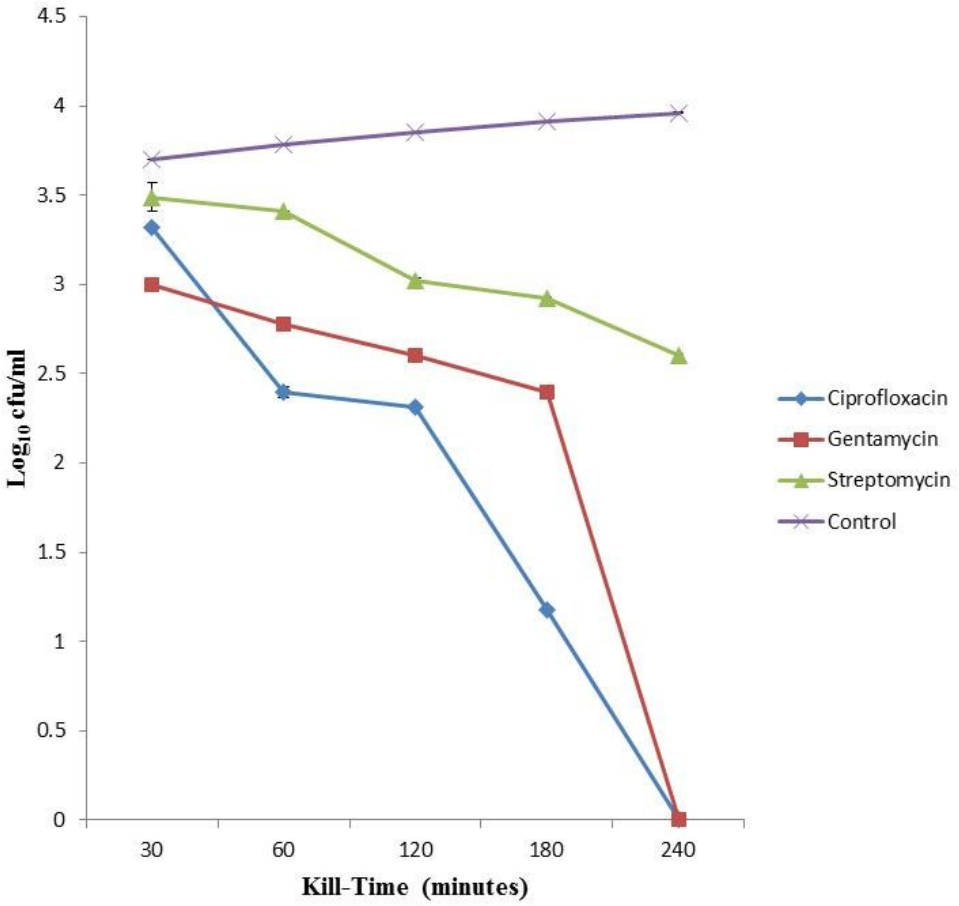

Figure 1: Population Density of *C. sakazakii* (log_{10}cfu/ml) versus Kill-Time (minutes)

C. sakazakii between 30-240 minutes (Figure 1). This result further support the antibiotic susceptibility profile carried out before the time-kill using the three antibiotics (Ciprofloxacin, Gentamycin and Streptomycin) effective against *C. sakazakii*. The results of time-kill differ in different studies due to differences in the antibiotics used, organisms used and variation in timing (Peter *et al.*, 2007). Determination of time-kill is very important in clinical settings as it guides in the duration of drugs usage and treatment of diseases especially in some clinical settings (Fung-Tomc *et al*., 2000). The ability of Ciprofloxacin and Gentamycin to kill *C. sakazakii* at 240 minutes makes them bactericidal and possibly makes them effective and potent in killing *C. sakazakii* than Streptomycin. On the other hand, a steady increment in the *C. sakazakii* populations from 30-240 minutes in the control were expected as no antibiotic was used in the control (Figure 1). This shows that the absence of antibiotics in the control facilitated the steady growth of *C. sakazakii* while the use of antibiotics against *C. sakazakii* either inhibit or kill it (bacteriostatic or bactericidal) as shown in Figure 1.

CONCLUSION

The results of the antibiotic susceptibility profile revealed that the isolates were susceptible to Ciprofloxacin, Gentamycin and Streptomycin in this order and the time-kill curve showed that Ciprofloxacin and Gentamycin killed *C. sakazakii* at 240 minutes making them better than the other antibiotics used in this study. This study showed the importance of selecting the right antibiotics to be used against the infections caused by *C. sakazakii* and the need to determine time-kill of antibiotics against organisms which is very relevant in disease management.

ACKNOWLEDGMENTS

The authors would like to appreciate God for the success recorded in the study. We also wish to thank the following people for their immense contributions to the study: Late Prof. Damisa Duro (Department of Microbiology, Federal University of Technology, Minna, Niger State); Prof. Evans Egwin (Department of Biochemistry, Federal University of Technology, Minna, Niger State); Dr. Abdulsalam Ihimma (Department of Applied Science, Kaduna Polytechnic, Kaduna, Kaduna State); Prof (Mrs) Abimbola Orukotan (Department of Microbiology, Kaduna State University, Kaduna, Kaduna State) and Mallam Sani Mohammed (Department of Microbiology, Kaduna State University, Kaduna, Kaduna State).

REFERENCES

Aigbekaen, B. O. and Oshoma, C. E. (2010). Occurrence of *Enterobacter sakazakii* from Powdered Foods Locally Consumed in Nigeria. *Pakistan Journal of Nutrition,* 9 (7): 659-663.

Aisha, M., Gbonjubola, O. A. and Yakubu, K. I. (2013). Incidents and antibiotic susceptibility pattern of bacteria isolates from wound infections in tertiary hospital Nigeria. *Tropical journal of pharmaceutical research*, 12 (4): 617-621.

Bowen, A., Wiesenfeld, H. C., Kloesz, J. L., Pasculle, A.W., Nowalk, A. J., Brink, L., Elliot, E., Martin, H. and Tarr, C.L. (2017). Notes from the Field: *Cronobacter sakazakii* infection associated with feeding extrinsically contaminated expressed human milk to a premature infant- Pennsylvania, 2016. *Morbidity* and *Mortality Weekly Report*, 66: 761

Brooks, G. F, Carroll, K. C., Butel, J. S. and Morse, S. A. (2007). Microscopy and Stains. In: Jawetz, Melnick and Adelberg's *Medical Microbiology*. Twenty-fourth Edition (pp.700-701). U.S.A: The McGraw-Hill Companies, Inc.

Centre for Disease and Control (2001). *Enterobacter sakazakii* infections associated with the use of powdered infant formula-Tennessee. *Morbidity* and *Mortality Weekly Report,* 51(14): 298-300.

Centres for Disease Control and Prevention (2015). Cronobacter: Expanded Information. Available online: *https://www.cdc.gov/cronobacter/technical.html* (accessed on 3rd February, 2019).

Cheesbrough, M. (2010). Laboratory Manual. In: *District Laboratory Practice in Tropical Countries* (pp.146-157). United Kingdom: Cambridge University Press.

Clinical and Laboratory Standards Institute (2015). *Performance Standards for Antimicrobial Susceptibility Testing; Twenty -Fifth Informational Supplement*. CLSI document M100-S25, Volume 35, No. 3.950 West Valley Road, Suite 2500 Wayne, Pennsylvania 19087 USA.

.

Ezeh, A. R., Tall, B. D., Gangiredla, J., Smith, S. I. and Olusimbo O. Aboaba, O. O. (2018). Determination of the Phylogenetic Relatedness of *Cronobacter Spp*. Isolated from Powdered Infant Formula Retailed in Nigeria using Pan Genomic DNA Microarray. *International Journal of Research - Granthaalayah,* 6(7): 327-340.

Feeney, A., Kropp, K. A., O'Connor, R. and Sleator, R. D. (2014). *Cronobacter sakazakii*: stress survival and virulence potential in an opportunistic food borne pathogen. *Gut Microb*es 5: 711–718.

Fung-Tomc, J. C., Gradelski, E., Valera, L., Kolek, B. and Bonner, D. P. (2000). Comparative Killing Rates of Fluoroquinolones and Cell Wall-Active Agents. *Antimicrobial Agents and Chemotherapy*, 44 (5): 377–1380.

Gengo, F. M., Mannion, T. W., Nightingale, C. H. and Schentag, J. J. (1984). Integration of pharmacokinetics and pharmacodynamics of methicillin in curative treatment of experimental endocarditis. *Journal of Antimicrobial Chemotherapy*, 14: 619-631.

Hardy Diagnostics Manual (2011). HardyCHROM sakazakii Medium-Microbiology Culture Media (pp.1-5). USA: 1430, West McCoy Lane, Santa Maria, CA93455.

Henry, M. and Fouladkhah, A. (2019). Outbreak History, Biofilm Formation, and Preventive Measures for Control of *Cronobacter sakazakii* in Infant Formula and Infant Care Settings. *Microorganisms*, 7: 77.

Hugo, W. B. and Russell, A. D. (1998). Determination of Rate of Killing. In: *Pharmaceutical Microbiology*. 6th Edition (pp. 91-100). Oxford: Clarendon Press.

Hunter, C. J. and Bean, J.F. (2013). Cronobacter: An emerging opportunistic pathogen associated with neonatal meningitis, sepsis and necrotizing enterocolitis. *JournalPerinatolology*, 33: 581.

Jackson, E. E., Flores, J. P., Fernandez-Escartin, E. and Forsythe, S. J. (2015). Reevaluation of a suspected *Cronobacter sakazakii* outbreak in Mexico. *Journal of Food Prot*ection, 78: 1191–1196.

Jawad, A., Amjad, A., Masroor, H. and Habib, A. (2013).Antimicrobial susceptibility pattern of bacteria isolated from burn wounds in a tertiary care hospital in *Pakistan. African Journal of Microbiology Research*, 7(28): 3627-3631.

McFarland, J. (1907). Nephelometer. *JAMA,* 14: 1176-1178.

Mohammedaman, M., Alemseged, A. and Tsegaye, S. (2014). Antimicrobial susceptibility pattern of bacterial isolates from wound infection and their sensitivity to alternative topical agents at Jimma University Specialized Hospital, South-West Ethiopia. *Annals of clinical Microbiology and Antimicrobial*, 13: 14.

Murray, P. R., Baron, E. J., Jorgensen, J. H., Landry, M. L. & Pfaller, M. A. (2007). Manual of Clinical Microbiology. Ninth Edition (pp. 2082-2091). Washington DC: ASM Press

Peter, J. P., Hal Jones, C. and Patricia, A. B. (2007). In vitro antibacterial activities of tigecycline and comparative agents by time-kill kinetic studies in fresh Mueller-Hinton broth. Infectious Disease Discovery Research, Biological Technologies, Wyeth Research, Pearl River, NY 10965, USA.

Smirnova, G. V. and Oktyabrsky, O. N. (2018). Relationship between *Escherichia coli* growth rate and bacterial susceptibility to ciprofloxacin. *Federation of European Microbiological Societies Microbiology Letters*, 365 (1): 1-6.

Stock, I. and Wiedemann, B. (2002). Natural antibiotic susceptibility of *Enterobacter amnigenus, Enterobacter cancerogenus, Enterobacter gergoviae* and *Enterobacter sakazakii* strains. *Clinical Microbiology Infections,* 8: 564-78.

Willey, M. J., Sherwood, M. L. and Woolverton, C. J. (2011). Antimicrobial Sensitivity. In: *Prescott's Microbiology*. Eight Edition (pp. 806-819). New York: McGraw-Hill, Companies, Inc.

World Health Organisation (2004). *Enterobacter sakazakii* and other microorganisms in powdered infant formula. MRA Series 6.