

Comparative *In Vitro* Activity of Ceftazidime Alone and Ceftazidime/Sulbactam against ESBL Producing Gram Negative Isolates from a Tertiary Care Hospital. A Study from Ahmednagar, Maharashtra

Prasad Niranjana Gunjal* and Shraddha Prasad Gunjal

Department of Microbiology, DVVPPF's Medical College, Maharashtra, India

*Corresponding Author: Prasad Niranjana Gunjal, Department of Microbiology, DVVPPF's Medical College, Maharashtra, India.

Received: January 12, 2018; Published: February 15, 2018

Abstract

An effective strategy to combat against the specific mechanism of resistance showed by the extended spectrum beta lactamase (ESBL) producing gram negative bacilli (GNB) is to use beta-lactamase inhibitor in combination with beta lactam antimicrobial drugs against these isolates of GNB's. The treatment of patients suffering with severe infections due to ESBL producers can be achieved by proper studies showing analysis and comparison of available options of combinations of drugs. The current study compares the *in vitro* activity of ceftazidime alone with ceftazidime/sulbactam in combination against such isolates of ESBL producing GNB's, the major threat for the hospitalized patients especially in developing and under developed countries.

Conclusion: The study concludes that ceftazidime although being an effective third generation cephalosporin, but due to emergence of resistant strains is losing the effectivity and hence the need to use a better option of combination of beta-lactam antibiotic with a beta-lactamase inhibitor such as ceftazidime/sulbactam.

Keywords: Ceftazidime; Sulbactam; Extended Spectrum Beta Lactamase (ESBL); Gram Negative Bacilli

Introduction

In today's world an important public health hazard amongst developed as well as developing countries are hospital acquired infections (HAI). The reasons of large number of causality in hospitalized patients are due to HAI [1], which is prevalent upto 18.6% according to a survey depending on population and definitions of HAI used [2].

A range of gram negative organisms are mostly responsible for HAI like lower respiratory tract infections, urinary tract infections, pneumonia, wound infections, blood stream infections, surgical site infections and sepsis. The commonly isolated organisms are now are usually *Acinetobacter*, *Pseudomonas*, *E. coli*, *Klebsiella*, *Enterobacter*, etc [3-8].

The increasing drug resistance among such gram negative isolates due to particular reasons [9-11].

Due to its proven safety, efficacy, broad spectrum activity a well characterized pharmacokinetic and pharmacodynamics properties. In our clinical settings, the routinely used β -lactam antibiotics are third generation cephalosporins, such as ceftazidime, cefotaxime and ceftriaxone [12].

In 1980's ceftazidime was introduced in clinical use due to broad spectrum activity against gram positive cocci (GPC) and gram negative bacilli (GNB). The activity against GNB has become compromised over a period of time due to the ESBL producing pathogens. These ESBL producing pathogens has become a grave problem in hospital acquired infection patients today [13]. Such a wide spread dissemi-

nation of bacterial drug resistance to a variety of β -lactam antibiotics possess a serious threat to effective use of these antibiotics, low permeability, and over expression of efflux pump and biofilm formation [14].

Current resistance to ceftazidime ranging from 30 - 71% [9,15,16] according to various surveillance reports across globe. In such scenario theoretical combination therapy should be reinforced. When there is no other drug to treat. The current trend is to use β -lactam antibiotics in combination with β -lactamase inhibitor such as sulbactam [17].

Sulbactam a β -lactamase inhibitor competitive irreversible and has effective inhibitory activity against clinically important plasmid mediated β -lactamase which are frequently responsible for transferable drug resistance [18]. Including India sulbactam a β -lactamase inhibitor is approved in many countries to be used in combination with β -lactam antibiotics [19]. Treating infections of ESBL producing GNB isolates with ceftazidime in combination with β -lactamase inhibitor like sulbactam would be a strong basis of rational therapeutics [18].

Study suggests combination of β -lactamase inhibitor and β -lactam antibiotics should be encouraged for further *in vivo* studies as that may come up with a better therapeutic outcome for HAI patients and to save life of such patients suffering with life threatening infections.

Materials and Methods

The study was carried out at Dr. Vitthalrao Vikhe Patil Foundation's Medical College and Hospital, Ahmednagar, Maharashtra. Study period was October 2012 to January 2013.

Methodology

Inclusion criteria

Samples were selected randomly and multiple but different isolates from single specimen were also considered.

Sr. No.	Nature of Specimens	Total Number of Clinical Specimens (N = 186)	Growth in Total Number of Specimens (N = 72) (%)
1	Pus	58	30 (16.30%)
2	Urine	45	16 (8.70%)
3	Fluid	28	12 (5.98%)
4	Sputum	28	12 (5.98%)
5	Blood	27	03 (1.63%)
Total		186 (100%)	72 (39.13%)

Table 1: Distribution of randomly selected clinical specimens including gram positive and gram negative isolates.

Methodology of testing

All the specimens were cultured and isolates were identified by standard methods of identification [20]. Isolates for production of enzyme Extended Spectrum Beta Lactamase (ESBL) were determined by applying ceftazidime and ceftazidime/clavulanic acid discs as per the CLSI guidelines [21]. The antimicrobial drug sensitivity detection testing was carried out as per the standard guidelines of CLSI using the Kirby Bauer's disc diffusion method. The discs of ceftazidime and ceftazidime/sulbactam combination were provided by Venus Remedies Ltd., Baddi (H.P.). These discs were manufactured by Hi-Media Laboratories, Mumbai as per the CLSI standard parameters.

The disc of ceftazidime and ceftazidime/sulbactam combination each contain 30 μ g of respective antibiotics. The break points for antimicrobial sensitivity testing interpretation of ceftazidime were according to CLSI guideline and that of the ceftazidime/sulbactam was provided by Venus Research Center.

All the isolates were lawn cultured using inoculums matched with Mac-Farland’s standard tube no. 5, for the turbidity match of inoculum. Muller-Hinton Agar (MH Agar) plates were used for performing the antimicrobial drug sensitivity detection testing by Kirby Bauer’s disc diffusion method. All plates were kept for incubation at 37°C for 18 hrs. ATCC *E. coli* strain No. 35218 and ATCC strain No. 27853 of *Pseudomonas aeruginosa* were used as internal quality control strains. After end of incubation phase all plates were examined and zone of inhibition readings were taken under three headings:

- A. **Sensitive:** Showing wide zone of inhibition of growth of test isolate indicating sensitivity.
- B. **Intermediate Sensitive:** Showing narrow zone of inhibition of growth of the test isolate indicating intermediate sensitivity.
- C. **Resistant:** Showing no zone of inhibition of growth up to margins of the disc of the test isolates indicating resistance.

Note: For convenience intermediate sensitive strains were also included into resistant strains.

All readings were noted according to reference ranges provided by manufactures for both the antibiotics.

Results and Observations

This table shows the total distribution of isolates of gram negative bacilli from all clinical specimens. Table 2, explains that from total 186 clinical specimens 66 i.e. 35.48% isolates were of gram negative organisms. Out of these 66 (35.48%) 19 isolates (28.78%) were of *E. coli* and *Klebsiella pneumoniae* respectively, 16 (24.24%) isolates were of *Pseudomonas aeruginosa*, 5 (7.57%) isolates of *Acinetobacter spp.*, 4 (6.06%) isolates were of *Citrobacter frundii*, 2 (3.03%) isolates were of *Proteus mirabilis* and 1 (1.57%) of *Proteus vulgaris* were isolated.

Sr. No.	Isolates	Pus N = 27	Urine N = 16	Fluid N = 13	Sputum N = 9	Blood N = 00	Total N = 66
1	<i>Pseudomonas aeruginosa</i>	07	02	04	03	00	16
2	<i>Acinetobacter spp.</i>	01	01	02	01	00	05
3	<i>E. coli</i>	09	06	03	01	00	19
4	<i>Klebsiella pneumoniae</i>	07	04	03	04	01	19
5	<i>Proteus mirabilis</i>	01	01	00	00	00	02
6	<i>Proteus vulgaris</i>	00	01	00	00	00	01
7	<i>Citrobacter frundii</i>	02	01	01	00	00	04
Total		27	16	13	09	01	66

Table 2: Number of gram negative organisms isolated from clinical specimens.

Table 3, explains about the isolates of GNB’s isolated from all clinical specimens were about 66 i.e. 35.48%.

Sr. No.	Nature of Specimens	Total Number of Specimens (N = 186) (%)	Total Number of Gram Negative Bacilli Isolated (N = 66) (%)	Total Number of ESBL Producing Gram Negative Bacilli Isolated (N = 38) (%)
1	Pus	58 (31.18)	27 (46.55)	18 (66.66)
2	Urine	45 (24.19)	16 (35.55)	13 (81.25)
3	Fluid	28 (15.05)	13 (46.42)	02 (15.38)
4	Sputum	28 (15.05)	09 (32.14)	05 (55.55)
5	Blood	27 (14.51)	01 (3.70)	00 (00.00)
Total		186 (100)	66 (35.48)	38 (57.57)

Table 3: Distribution of isolates detection producing ESBL and without production of ESBL in total gram negative isolates from all specimens.

Total number of pus specimens were 58 i.e. 31.18% out of total 186 specimens included in the study. Out of these 58 i.e. 31.18% of pus specimens GNB were isolated from total 27 i.e. 46.55% of specimens and out of these 27 i.e. 46.77% isolates of pus GNB total 18 i.e. 66.66% GNB were detected to be ESBL producer pathogens.

Out of 186, urine specimens included were 45 specimens i.e. 24.19%, in these 45 i.e. 24.19% of GNB isolated from total 16 specimens i.e. 35.55% and among these 16 GNB isolates i.e. 35.55% total 13 i.e. 81.25% isolates were detected as ESBL producing pathogens.

Total 28 body fluid as well as sputum specimens out of 186 specimens of study group were received during study period and out of 28 i.e. 15.05% specimens of body fluid 13 i.e. 46.42% were showed growth of GNB and out of these 13 i.e. 46.42% of GNB's total 02 were detected as ESBL producers i.e. 15.38%. While in the sputum total 09 were isolates of GNB i.e. 32.14% and out of these 09 i.e. 32.14% total 05 were detected as ESBL producer i.e. 55.55%.

Total specimens for blood culture received during study period were 27 i.e. 14.51% out of 186 specimens. Out of these 27 i.e. 14.51% specimens only a single specimen is grown with GNB i.e. 3.70% and the same was not detected as a ESBL producer one.

Out of total 66 isolates of GNB amongst all the 186 specimens. This table 4 describes about distribution of organism wise isolates as well as organism wise ESBL producers.

Sr. No.	Isolates	Total Number of Isolates (%) (N = 66)	Total Number of Isolates Detected as ESBL Producers (%) (N = 38)
1	<i>Pseudomonas aeruginosa</i>	16 (24.24)	07 (18.42%)
2	<i>Acinetobacter spp.</i>	05 (7.57)	02 (5.26)
3	<i>E. coli</i>	19 (28.78)	14 (36.84%)
4	<i>Klebsiella pneumoniae</i>	19 (28.78)	11 (28.94)
5	<i>Proteus mirabilis</i>	02 (3.03)	01 (2.63)
6	<i>Proteus vulgaris</i>	01 (1.51)	01 (2.63)
7	<i>Citrobacter freundii</i>	04 (6.06)	02 (5.26)
Total		66 (100)	38 (57.57)

Table 4: Distribution of number of ESBL producing gram negative bacilli (GNB) isolated from clinical specimens.

Out of the 66 (100%) gram negative bacilli isolates, 38 (57.57%) were detected as ESBL producer amongst which *E. coli* and *Klebsiella pneumoniae* isolates were total 19 (28.78%) respectively and 14 (36.84%) of *E. coli* and 11 (28.94%) of *Klebsiella pneumoniae* were detected as ESBL producer.

Amongst 66 (100%) gram negative bacilli isolates, 16 (24.24%) were the isolates of *Pseudomonas aeruginosa*. In 16 (24.24%) of these isolates 07 (18.42%) were detected as ESBL producer.

Isolates of *Acinetobacter spp.* and *Citrobacter freundii* were 05 (7.57%) and 04 (6.06%) respectively and in both the organisms 02 i.e. (5.26%) isolates were detected with the production of enzyme ESBL.

Amongst the *Proteus mirabilis* and *Proteus vulgaris* isolates 02 (3.03%) and 01 (1.51%) respectively, only 01 i.e. (2.63%) were detected as ESBL producer.

Table 5, describes about the resistance pattern of the isolates shown towards ceftazidime alone and ceftazidime/sulbactam combination out of total 38 (57.57%) of 66 gram negative isolates detected as ESBL producer. Amongst all these 38 (100%) isolates 31 (81.57%) had showed resistance to ceftazidime alone and 10 (26.31%) were detected as resistant to ceftazidime/sulbactam combination.

Sr. No.	Isolates	Total Number of Isolates Detected as ESBL Producer (N = 38) (%)	Total Number of Isolates Showed Resistance to Ceftazidime Disc (30 µg) (N = 31) (%)	Total Number of Isolates Showed Resistance to Ceftazidime/Sulbactam Combination (30 µg) (N = 10) (%)
1	<i>Pseudomonas aeruginosa</i>	07 (18.42)	05(71.42)	01 (14.28)
2	<i>Acinetobacter spp.</i>	02 (5.26)	02 (100)	01 (50)
3	<i>E.coli</i>	14 (36.84)	13 (92.85)	03 (21.42)
4	<i>Klebsiella pneumoniae</i>	11 (28.94)	09 (81.81)	04 (36.36)
5	<i>Citrobacter frundii</i>	02 (5.26)	02 (100)	01 (50)
6	<i>Proteus mirabilis</i>	01 (2.63)	00 (00)	00 (00)
7	<i>Proteus vulgaris</i>	01 (2.63)	00 (00)	00 (00)
Total		38	31	10

Table 5: Resistance pattern of isolates to ceftazidime alone and ceftazidime/sulbactam in combination.

Pseudomonas aeruginosa isolates 05 (71.42%) were showed resistance to ceftazidime alone whole 1 (14.28%) only had shown resistance to ceftazidime/sulbactam combination.

Amongst 2 (5.26%) isolates of *Acinetobacter spp.* 2 (100%) showed resistance to ceftazidime alone while only 01 (50%) had showed resistance to ceftazidime/sulbactam combination.

E. coli isolates detected as ESBL producer were 14 (36.84%) amongst total 38 (100%) isolates of ESBL producer of GNB. In these 14 (100%) isolates 13 (92.85%) showed resistance to ceftazidime alone and among the 14 (100%) ESBL producer of *E. coli* only 3 (21.42%) had showed resistance to ceftazidime/sulbactam combination.

Total 9 (81.81) isolates of *Klebsiella pneumoniae* showed resistance to ceftazidime alone while 4 (36.36%) showed resistance to ceftazidime/sulbactam combination, 2 (100%) isolates of *Citrobacter frundii* were detected as resistant to ceftazidime alone in them 1 (50%) detected as resistant to ceftazidime/sulbactam combination.

In the isolates of *Proteus mirabilis* and *Proteus vulgaris* total 1 (2.36%) respectively detected as ESBL producer the isolates showed susceptibility towards both the drugs ceftazidime and ceftazidime/sulbactam combination.

Discussion

The greatest achievements of modern medicine is discovery and development of antimicrobial agents, but in today’s world this achievement and its effectiveness is being challenged by microbes due to their ability to re-emerge with new form of enzyme and drug resistance characters such as mutation [22,23].

In our study shows increased susceptibility to ceftazidime/sulbactam combination by all the ESBL producing gram negative isolates. This susceptibility may be due to the sulbactam significantly potentiating ceftazidime against gram negative isolates [1].

Similar findings have been reported by Manu Chaudhary, *et al.* [1], that about 93.4% susceptibility to ceftazidime/sulbactam shown by *Pseudomonas aeruginosa*, while 86.3% by *E. coli*, whereas in our study the 14.28% isolates shown resistance to ceftazidime/sulbactam combination while 21.42% of *E. coli* isolates showed resistance to this combination.

Wahid, *et al.* [18], demonstrated 34.29% of resistance to the combination of ceftazidime/sulbactam while in our study this resistance is 26.31% only. Which shows a reduced rate of resistance amongst the isolates as the pervious study was our piolet study and the reduction in rate of resistance may be due to the awareness amongst the hospital staff about hospital acquired infections after publishing the first data.

Zang and Li [24], also reported that combination of sulbactam and ceftazidime at the ratio of 1:1, ceftazidime resistant isolates become sensitive to ceftazidime/sulbactam combination, this pattern of susceptibility has also been seen in our study.

Kolayl, *et al.* [25], states that ceftazidime/sulbactam may be a reasonable alternative to carbapenems in empirical regimen.

Study denotes toward high resistance to ceftazidime alone amongst all the ESBL producing GNB's, this finding is similar to the previous studies reported, in which also it is reported that susceptibility of third generation cephalosporin is decreased against *Pseudomonas* and other non-fermenters like *Acinetobacter spp.* as well as amongst the isolates of *Enterobacteriaceae* family like *E. coli*, *Klebsiella pneumoniae*, *Citrobacter freundii*, etc. One of the most important reasons for this resistance in various mechanisms of resistance is exhibited by GNB's is ESBL production [26-29].

Kumar, *et al.* [23] documented resistance of 35.55% by ESBL producing *E. coli* while in our study this resistance is 21.42% which is in well accordance with the study.

Conclusion

From the above study data, it is evident that ceftazidime alone losing the combat against the ESBL producing resistant strains isolated in hospital setting responsible for hospital acquired infections. Hence the need for effective treatment option and this option could be the Beta-lactam and Beta-lactamase inhibitor like ceftazidime/sulbactam combination. This combination has showed an enhanced *in vitro* activity against all the ESBL producing isolates in comparison with ceftazidime alone. Therefore ceftazidime/sulbactam can be a better option for treatment especially isolates resistant to third generation cephalosporins.

So to conclude this combination could be a better alternative only after successful clinical trials. And also similar studies should be encouraged to overcome the present situation of combating against the resistant strains in hospital settings.

Acknowledgement

Authors acknowledge the Principal and Management of Dr. Vithalroa Vikhe Patil Foundation's Medical College and Hospital, Vilad Ghat, Ahmednagar, Maharashtra, India, for support to carry out the study.

Bibliography

1. Chaudhary M and Payasi A. "Comparative *in Vitro* Activity of Zydorum against Gram Negative and Gram Positive Clinical Isolates". *IOSR Journal of Dental and Medical Sciences* 14.4 (2015): 74-78.
2. Hughes AJ, *et al.* "Prevalence of nosocomial infection and antibiotic use at a university hospital in Malaysia". *Infection Control and Hospital Epidemiology* 26.1 (2005): 100-104.
3. Mordi RM and Momoh MI. "Incidence of Proteus species in wound infections and their sensitivity pattern in the University of Teaching Hospital". *African Journal of Biotechnology* 8.5 (2009): 725-730.
4. Kamath S, *et al.* "Nosocomial infections in neonatal intensive care units: profile, risk factor assessment and antibiogram". *Indian Journal of Pediatrics* 77.1 (2007): 37-39.
5. Blomgran R, *et al.* "Uropathogenic Escherichia coli trigger oxygen-dependent apoptosis in human neutrophils through the cooperative effect of type I fimbriae and lipopolysaccharide". *Infection and Immunity* 72.8 (2004): 4570-4578.
6. Jha N and Bapat SK. "A study of sensitivity and resistance of pathogenic microorganisms causing UTI in Kathmandu Valley". *Kathmandu University Medical Journal* 3.2 (2005): 123-129.
7. Hare NJ, *et al.* "Proteomic of Pseudomonas aeruginosa AES-IR, PAO1 and PA14 reveals potential virulence determinants associated with a transmissible cystic fibrosis-associated strain". *BMC Microbiology* 12 (2012): 16.

8. Vincent JL, *et al.* "International study of prevalence and outcomes of infection in intensive care units". *Journal of the American Medical Association* 302.21 (2009): 2323-2329.
9. Neuhauser MM, *et al.* "Antibiotic resistance among gram-negative bacilli in US intensive care units. Implications for fluoroquinolone use". *Journal of the American Medical Association* 289.7 (2003): 885-888.
10. Mutlu M, *et al.* "Neonatal sepsis caused by Gram negative bacteria in a neonatal intensive care unit: a six years analysis". *Hong Kong Journal of Paediatrics* 16 (2011): 253-257.
11. Niranjan V and Malaini A. "Antimicrobial resistance pattern in Escherichia coli causing urinary tract infection among inpatients". *Indian Journal of Medical Research* 139.6 (2014): 945-948.
12. Andes DE and Craig WA. "Cephalosporins". In: Mandell GL, Bennett JE, Dolin R, editors, Mandell, Douglas and Bennett's Principles and practice of Infectious Diseases. 7th edition Philadelphia. PA: Churchill Livingstone Elsevier (2010): 323-337.
13. Pitout JD. "Infections with extended spectrum beta-lactamase-producing enterobacteriaceae: changing epidemiology and drug treatment choices". *Drugs* 70.3 (2010): 313-333.
14. Kalantar E, *et al.* "Incidence and susceptibility pattern of metallo-beta-lactamase producers among Pseudomonas aeruginosa isolated from burn patients at Kurdistan province". *Jundishapur Journal of Microbiology* 5.3 (2012): 507-510.
15. Turner PJ. "Mystic Europe 2001: activity of meropenem and other broad spectrum agents against nosocomial isolates". *Diagnostic Microbiology and Infectious Disease* 63.2 (2009): 217-222.
16. Aurangezeb B and Hameed A. "Neonatal sepsis in hospital-born babies: bacterial isolates, antibiotic susceptibility patterns". *Journal of College of Physicians and Surgeons Pakistan* 13.11 (2003): 629-632.
17. Livermore DM and Woodford N. "The Beta-lactamase threat in Enterobacteriaceae, Pseudomonas and Acinetobacter". *Trends in Microbiology* 14.9 (2006): 413-420.
18. Ambekar AW, *et al.* "In vitro activity of ceftazidime/sulbactam and cefepime/sulbactam in combination against ESBL producing isolates". *Undergraduate Journal of Psychology at Berkeley* 2.1 (2014): 25-58.
19. Durajraj S, *et al.* "Simultaneous estimation of ceftriaxone sodium and sulbactam sodium using multicomponent mode of analysis". *International Journal of ChemTech Research* 2.4 (2010): 2177-2181.
20. Washington W, *et al.* "Koneman's color atlas and text book of diagnostic microbiology". Sixth edition. Lippincott Williams and Wilkins. The role of microbiology laboratory diseases: Guidelines to practice and management (2006): 2-6.
21. Mitchell J Schwaber, *et al.* "Utility of NCCLS guidelines for identifying extended -spectrum beta-lactamase in non-Escherichia coli and non-Klebsiella spp. of Enterobacteriaceae". *Journal of Clinical Microbiology* 42.1 (2004): 294-298.
22. Vidushi KC, *et al.* "Comparative analysis of antimicrobial susceptibility profile of drug resistant and susceptible strains of gram negative bacteria". *World Journal of Pharmaceutical Research* 6805.5 (2016): 1543-1553.
23. Kumar D, *et al.* "Antimicrobial Susceptibility Profile of Extended Spectrum [beta]-Lactamase (ESBL) Producing Escherichia coli from Various Clinical Samples". *Infectious Diseases* 7 (2014): 1-8.
24. Zang YL and Li JT. "The in vitro activity of sulbactam combined with third generation cephalosporins against third generation cephalosporin-resistant bacteria". *International Journal of Antimicrobial Agents* 17.2 (2001): 143-146.
25. Koyal F, *et al.* "In vitro activity of oxymino-cephalosporins with and without sulbactam against Class A Extended-spectrum-beta-lactamase producing E. coli". *Journal of Microbiology and Infectious Diseases* 1.3 (2011): 87-92.

26. Garau G., *et al.* "Structure-based phylogeny of the metallo-beta-lactamase". *Antimicrobial Agents and Chemotherapy* 49.7 (2005): 2778- 2784.
27. Gunjal PN and Gunjal SP. "Assessment of sensitivity pattern of cefepime alone and cefepime/sulbactam in combination, against ESBL producing Gram negative isolates from a tertiary care hospital. A study from Ahmednagar, Maharashtra". *World Journal of Pharmacy and Pharmaceutical Sciences* 4.8 (2015): 743-750.
28. Gunjal PN., *et al.* "A cross sectional study to determine the profile and antibiotic resistance pattern of gram neative bacilli isolated from intensive care unit patients in a tertiary care hospital in Ahmednagar, Maharashtra". *International Journal of Biomedical Research* 3.5 (2012): 281-284.
29. Gunjal SP., *et al.* "Carbapenem resistance profile amongst Escherichia coli and Klebsiella pneumoniae in a tertiary care hospital in Ahmednagar, Maharashtra". *International Journal of Medical Research and Health Sciences* 3.2 (2014): 424-427.

Volume 14 Issue 3 March 2018

©All rights reserved by Prasad Niranjana Gunjal and Shraddha Prasad Gunjal.