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# Bioprospecting of Horseshoe Crabs and Biotechnological Approaches Towards Sustainable Utilization

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## ABSTRACT

*Horseshoe crabs are unique type of arthropod having a lot of biomedical and evolutionary significance. Only four species of horseshoe crabs are presently available throughout the globe. In India two species of horseshoe crabs namely Tachypleus gigas and Carcinoscorpius rotundicauda have been reported from estuaries of Odisha and west Bengal coast. Besides the ecological significance they have immense biological, biomedical, economical and biotechnological prospects. The specific proteins present in the cells of blue blood of such animals have been used for detection of minute quantity of endotoxins. The chitin and chitosan of horseshoe crabs carapaces have been used for various biomedical purposes. The proteins and other compound present in perivitelline fluid of horseshoe crabs reported to have stem cell growth promoters, cardiogenesis enhancement capacity etc. But due to natural climatic change and excessive human exploitation, their population is decreasing. Keeping view of their importance, certain biotechnological approaches have been developed recently to increase and sustain the population of horseshoe on the earth.*

*Keywords: Horseshoe crab, Chitin, Perivitelline fluid. Tachypleus gigas.*

## Introduction

Horseshoe crabs or King crabs are a type of Arthropod found in marine waters. They are not true crabs; rather more closely related to Arachnids, a group that includes spiders and scorpions. It is also known by various names in Odia like:

Samudra Bichha, Ram Laxman Kankada, Kankua, Kanku *etc.* There are four extant species of horseshoe crabs found globally namely: *Limulus polyphemous*, *Tachypleus tridentatus*, *Tachypleus gigas* and *Carcinoscorpius rotundicauda*. They are regarded as an oldest creature in the earth and are surviving from the Mesozoic era, before the dinosaurs came into existence, since 425 million years ago. Because of their origin more than 450 million years ago, horseshoe crabs are also considered as "living fossils".

## Bioresources

The blood and perivitelline fluid of eggs of horseshoe crabs have a number of biomedical important materials. Haemolymph or the blood of the Horseshoe crab is blue in colour, due to the presence of Haemocyanin. Plasma of the haemolymph contains the salt content of the sea water and has 3 abundant proteins i.e haemocyanin, the respiratory proteins and the C- reactive proteins which functions in the cytolytic destruction of bacterial cells. The protein isolated from the blood cells of American and Chinese horseshoe crabs have been used for development of very expensive and useful diagnostic kits (Lymulus Amoebocytes Lysate, LAL and Tachypleus Amoebocytes Lysate, TAL) for testing of the presence of bacterial endotoxins both Gram positive and Gram negative discovered by Levin *et al.* (1970). This test helps millions of human by providing safe and essential medical products. The America is getting huge revenue by supplying the product to different developed and developing countries. Indian has both the species of horseshoe crabs and therefore has enough scope to develop such kits for commercial uses. The lymph part of the horseshoe crab also contains some secondary metabolites and $B_{12}$ binding proteins. Haemolymph contains some large granules such as Factor–B, C, D and G, Proclotting enzyme, coagulogen, Anti-LPS factor, Big Defensins, Tachylectin 1-5, Cystatin, Alpha–2 macroglobulin, LICI-123 as well as some small granules such as Tachyplesins, Big Defensins, Tachycitins and Tachystatins (Shigenaga *et al.*, 1993) The Perivitelline fluid of the horseshoe crab also contains some important primitive type of proteins, such as hemagglutinins and haemocyanin which plays an important role during embryogenesis by promoting cell proliferation. Lectin is the constituent of the peri vitelline fluid that helps in the proliferation of Chick embryonic heart cells (Ghaskadbi *et al.*, 2008). In wound healing activity and differentiation of human bone marrow stem cell into cardiomyocyte (Huma *et al.*, 2015a and b). The crude PVF has the ability to induce differentiation of dendritic cells from its bone marrow precursors (Chinnari *et al.*, 2015). The role of such molecule for growth stimulating effect can generate millions of rupees and enrich the global economy including India in future.

## Potential Compounds for Anticancer Properties

The exploitation of new bio-active compounds from oceanic flora and fauna for its anti-carcinogenic properties has become an expanding biomedical field. This has lead to ever increasing quest to search for novel compounds with anti-cancer activity that could be used as drugs to cure the disease (Hoskin and Ramamoorthy, 2008). The naturally occurring antimicrobial peptides (AMPs), one of the efficient chemical defences of the eukaryotic cells against bacteria, fungi and viruses, have

recently received more attention due to its potential anti-cancer activity (Zasloff, 2002).

AMPs that selectively targets cancer cells without harming the normal mammalian cells, are of great advantage in clinical treatment (Hoskin and Ramamoorthy 2008). Among the known AMPs, tachyplesin I obtained from hemocytes of horseshoe crab is currently the upcoming new bio-active compound known for its unique method of killing the cancer cells (Nakamura *et al.*, 1988). Recently, a report demonstrated that tachyplesin I selectively bind to hyaluronan on hyaluronan overexpressing human TSU prostate carcinoma cells, leading to lysis of tachyplesin I-coated cancer cells via activating the complement pathway (Adamia *et al.*, 2005). Like many tumor cells, hyaluronan is also present in higher amounts on the surface of endothelial cells involved in the process of neovascularisation that occur during tumor development. This suggests that tachyplesin I might also cause destruction of neovascularisation as well as neoplastic cells via classical complement pathway (Rooney *et al.*, 1995). Moreover, another report showed that heat inactivated serum complement leads to partial survival of the tachyplesin I coated TSU prostate cancer cells from lysis (Chen *et al.*, 2001). This result thus, suggests that tachyplesin I is involved in another mechanism that can cause cancer cell death. Also, it has been shown that a synthetic tachyplesin conjugated to the integrin homing domain RGD inhibited the proliferation of cultured tumor and endothelial cells and also reduced the colony formation of TSU prostate cancer cells.

The same group further emonstrated that RGD-tachyplesin activates caspase 9, caspase 8, and caspase 3 and increase the expression of Fas, FasL and caspase 7, and caspase 6, indicating the role of RGD-tachyplesin in promoting mitochondria and Fas/FasL mediated apoptosis. The potential mechanism of role of RGD-tachyplesin suggests that the overall positive charge of tachyplesin I allow internalization of RGD-tachyplesin I to target and this disrupt the negatively charged membranes of the mitochondria in the cancer cells, and thus promotes apoptosis (Chen *et al.*, 2001). Further, tachyplesin I is shown to inhibit the cancer cell growth via a non-cytolytic mechanism (Hoskin and Ramamoorthy 2008). Upon treatment of tachyplesin I of SMMC-7721 human hepatoma cell and BGC-823 human gastric adenocarcinoma cell cultures decreases the proliferative capacity of these cells (Shi *et al.*, 2006). Also, growth of SMMC-7721 cells decreases in the presence of tachyplesin I and this was shown to be associated with the reversal of malignant morphological and ultrastructural features (Ouyang *et al.*, 2002). At molecular level, it was shown to decrease the expression of tumor-associated antigens such as α-fetoprotein, modulation of differentiation-associated enzyme such as glutamyltransferase expression. Also, it leads to the decrease of expression of the *c-myc* oncogene, and an increase in the expression of the tumor suppressor gene p21WAF1/CIP1 (Ouyang *et al.*, 2002). This result was also demonstrated in BGC-823 cells in the presence of tachyplesin I where the morphology and ultrastructure of these cancer cells were altered (Li *et al.*, 2000). Moreover, tachyplesin I inhibited *in vitro* proliferation, decreased the expression of *c-myc*, *c-erbB-2* and *mtp53* oncogene expression, and increasing *p16* tumor suppressor gene expression. Taken together, the above findings suggest that tachyplesin I is capable of inducing tumor cell differentiation, and

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thereby reversing the malignant phenotype. Tachyplesin I might down-regulate cathepsins in lysosomes and up-regulate TOP2A to inhibit migration and promote apoptosis in glioma, thus contribute to its anti-tumor function. Our results suggest tachyplesin I is a potential candidate for treatment of glioma (Xuan *et al.*, 2017).

## The Resources from Horseshoe Carapace

Chitin is a substance found in the shells, or exoskeleton, of horseshoe crabs. It is nontoxic and biodegradable and is processed to produce another substance called chitosan (aminopolysaccharide) that can be used to produce a variety of important products. Contact lenses, skin creams, and hair sprays can also be manufactured from chitin. Chitin can be used to remove lead and other harmful chemicals from wastewater. Chitosan also inhibits "bad" cholesterol uptake and boosts "good" cholesterol. Other chitosan uses include: promoting the healing of ulcers and lesions; serving in antibacterial action; acting as an antacid; helping to control high blood pressure; and treating and preventing irritable bowel syndrome (Pati *et al.*, 2018).

## Conclusion

Besides their ecological and evolutionary significance the horseshoe crabs have enormous potential resources for biomedical, commercial usages of the human welfare. This has also regarded as a valuable biological indicator of marine and estuarine environment. But there is an urgent need to understand the biology of this organism properly to protect them from extinction and for sustainable utilisation also.

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# Regional Pattern of Under-five Mortality in Odisha

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## Introduction

Childhood is a significant stage of life and deprivation during this period is known to have long-term adverse impact on the well-being of the individual during the adulthood and even during the old age. Reduction of the under-five morality by two third between 1990 and 2015 was one of the eight Millennium Development Goals of the United Nations Millennium Development Agenda which recognised that children were the most important assets of any nation. The decrease in the under-five mortality rate, therefore, is not only desirable but also indicative of an improvement in the level of social and economic development and general standards of living (Barman and Talukdar, 2014). The improvement in child survival has been found to be associated with positive changes in the socioeconomic status of the people (Houweling et al, 2007). In India, 2.1 million children are estimated to die before reaching their fifth birthday. Half of these children die even before they are 28 days old. Infant deaths in india account for one-fourth of the global infant deaths (Sharma, 2008). On the other hand, out of about 9.7 million child deaths worldwide annually, one-third occur in India because infants and children in India experience very high probability of death compared to probability of death in infants and children of many other developing countries (Rabbani and Qayyum, 2015). The probability of death in the first five-years of life in India is around 50 under-five deaths for every 1000 live births (International Institute for Population Sciences and IFC, 2017) which is about two times the probability of death during the first five years of life in many developing countries including Vietnam, Philippines and Indonesia (Westley, 2003). According to the World Health Organization, major causes of death during the first five years of life are prematurity, congenital sepsis, pneumonia, malaria, and birth complications (WHO, 2015). These morbid conditions are known to have strong association with a range of social and economic factors, such as poverty, education, especially, of women, and the place of residence (Das Gupta, 1990).

The infant mortality rate (IMR) is defined as the probability of a newborn dying before reaching the first birthday and is expressed as per 1000 live-births while under-five mortality rate (U5MR) is defined as the probability of a newborn dying before reaching the fifth birthday. Both the indicators have been used as measures of child well-being for many years. The level of under-five mortality is one of the most revealing reflections of how well the society is meeting the needs of its people, and how well Governments distribute assailable resources for health education, food distribution, sanitation, women's empowerment and other priorities in public spending. The International Conference on Primary Health Care held in Alma Ata in 1978 was the first global conference that highlighted how the risk of death in children can be reduced by the systematic development of the primary health care delivery system.

The U5MR varies widely across the states of India. The demographically less advanced states such as Bihar, Madhya Pradesh, Uttar Pradesh, Rajasthan and Odisha have very high levels of under-five mortality in the country. Under-five mortality needs a closer look when it comes to Odisha because Odisha is among the Empowered Action Group (EAG) states where the health and mortality situation is relatively poorer as compared to other states of the country (Chhavi and Mohanty, 2015). The IMR is Odisha is estimated to be 40 infant deaths for every 1000 live birth while the under-five mortality rate is estimated to be 44 under-five deaths for every 1000 live births in the year 2018 according to India's official sample registration system (Government of India, 2020). A range of complex and interrelated factors influence the U5MR. The socio-economic factors like that poverty, level of parental education, place of residence, occupations, income, safe drinking water facilities and improved sanitation have been found to have a strong impact on U5MR (Islam et al, 2013). Odisha is also the most poverty-stricken state of India with 47 per cent of the households living below the poverty line compared to 43 per cent in Bihar, 31 per cent in Uttar Pradesh, 37 per cent in Madhya Pradesh, 15 per cent in Rajasthan and 26 per cent in India as a whole (Pradhan and Arokiasamy, 2006). There are numerous studies which have examined the role of demographic factors such as woman's age at birth of the child, birth order and birth interval in deciding the level of U5MR in Odisha (Bennett,1999; Harden and Whittaker, 2011; Rabbani and Qayyum, 2015). One of the socio-economic determinants, mother's education, has been recognised as a powerful factor influencing child survival (Caldwell, 1979). There is a strong inverse relationship between mother's education and child mortality. Cessation of breastfeeding also has an impact on the risk of death during childhood, especially, infancy as cessation of breastfeeding leads of return of ovulation and hence conception so that the birth interval is short (Rafiqul et al,2013).

The U5MR in Odisha has been found to be higher in the rural as compared to the urban areas of the state because of better availability of health services, higher income and educational level in the urban areas. Significant cause of under-five deaths in rural Odisha are diarrhea, fever, respiratory infections and dropsy. Coverage of primary health care services including immunisation, institutional deliveries, etc. is better in the urban areas of the state as compared to its rural areas (Singh and Singh, 2017).

The foregoing considerations constitute the basis for the present study which attempts to analyse the factors that influence U5MR in Odisha through the regional perspective. The analysis is expected tp provide valuable insight as to why U5MR in the state remains so high. The paper also identifies factors that are responsible for the change in U5MR in the state during the last two decades.

## Data and Methodology

The data for the present analysis have been extracted from the Sample Registration System (SRS) and the National Family Health Survey (NFHS). The level, trend and regional differentials in U5MR have been analysed using SRS data. The Sample Registration System was introduced as a pilot scheme in some selected Indian states in 1964-65 to generate reliable estimates of fertility and mortality at national and state levels. It was converted into a full-scale system in 1969-70. On the other hand, determinants of U5MR have been analysed using the data available through the third and fourth rounds of NFHS.

The analysis was carried out using Stata software. Some variables included in the analysis were re-coded to construct new variables. All the variables used in the analysis were converted into categorical variables through appropriate coding. Descriptive statistics were applied to generate frequency tables and contingency tables. On the other hand, the Cox proportional hazard model was used for survival analysis. The model is defined as:

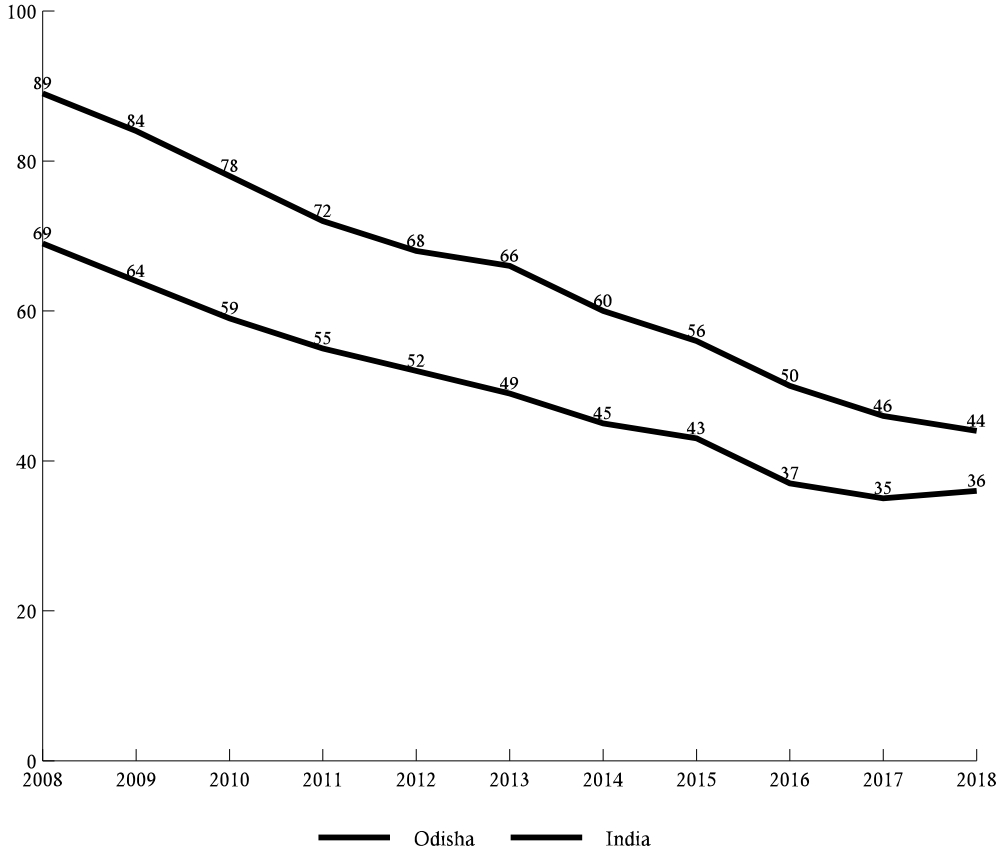
$$h_i(t_i|x_i) = h_0(t)\exp\left(\sum_{i=1}^{n}\beta_i x_i\right)$$

where $h_0(t)$ is the base line hazard. which can be approximated by any distribution such as exponential or Weibull distribution. In addition, the extension of the Blinder-Oaxaca decomposition technique (Fairlie 2005) has been used to analyse how the change in different explanatory factors contributed to the change in U5MR. The outcome or the dependent variable for the present analysis is a dichotomous variable which was assigned a value 1 if the newborn died before reaching 60 months of age and a value 0 if the newborn survived up to the 60 months of age or up to the fifth birthday.

## Findings

According to the latest estimates available through India's official sample registration system, the U5MR in Odisha was 44 under-five deaths for every 1000 live births in the year 2018 which was well above the national average of 36 under-five deaths for every 1000 live births (Government of India, 2020). The U5MR was marginally higher in male children as compared to female children of the state. In the rural areas of the state, there was virtually no difference between male and female U5MR but male U5MR is found to be higher than female U5MR in the

Figure 1
Trend in U5MR in Odisha and India

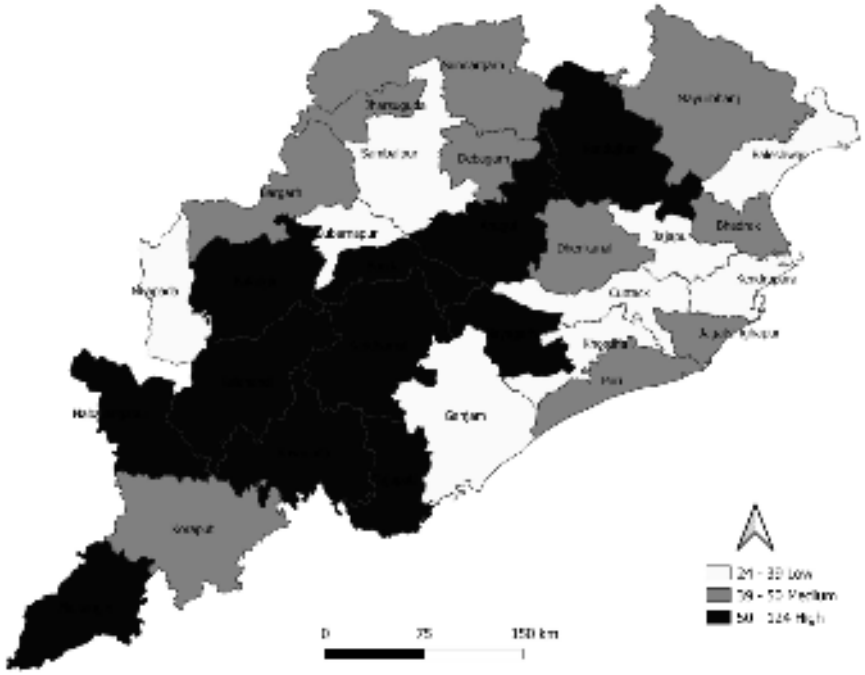



urban areas. On the other hand, U5MRis higher in rural than in urban areas of the state and the difference is substantial. For every 1000 live births, there are 10 more under-five deaths in the rural areas of the state as compared to the urban areas.

Estimates for the regions and districts of the state are obtained from the data available through NFHS 2015-16. Among different regions of the state, U5MR is estimated to be the highest in the southern region but the lowest in the central region. The U5MR in the southern region of the state is almost two times the U5MR in the central region (Table 1). In other regions of the state, the difference in U5MR is not very substantial and ranges between 45-50 under-five deaths for every 1000 live births according to the data available through NFHS 2015-16.

Table 1 also suggests that the U5MR varies widely by background characteristics in the state and in its different regions. For the state as a whole, U5MR is estimated to be the lowest in children of second birth order and U5MR increases sharply with the increase in birth order of the child. A similar pattern can also be seen in the western, northern and southern regions of the state but, in the central region, U5MR is the lowest in children of first birth order and increases with the increase in birth order. Similarly, male U5MR is higher than the female U5MR in the state and in its

Figure 2
Inter-district variation in U5MR in Odisha



southern and central regions but female U5MR is higher than the male U5MR in western and northern regions of the state. The U5MR is, however, substantially lower in children having at least 2.5 kg weight at the time of birth compared to children having less than 2.5 kg weight at birth.

For the state, U5MR is higher in rural as compared to urban areas but in the western region, U5MR is higher in the urban areas. On the other hand, U5MR decreases sharply with the increase in the level of education of the mother, exposure of the mother to the mass media and the standard of living as measured through the wealth index. Similarly, U5MR is found to be higher in children delivered in home compared to children delivered in an institution. The U5MR is also found to be the highest in Scheduled Tribes children but the lowest in children of Other Backward Classes but U5MR is the lowest in Scheduled Tribes children in the western region of the state.

District level estimates of U5MR derived from the data available through NFHS 2015-16 are presented in table 2. These estimates reflect wide variation in U5MR across the districts of the state ranging from 124 under-five deaths for every 1000 live births in district Malkangiri in the southern region of the state to only 24 under-five deaths for every 1000 live births in district Cuttack located in the central region of the state. In addition to district Malkangiri, district Raygada, also located in the southern region of the state, is the only district where the U5MR is estimated to be ore than 100 under-five deaths for every 1000 live births. The U5MR is also estimated to be very high in the Kandhmal district also located in the southern