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In Memoriam of Dr G. P. Sen

Late Prof. (Dr.) Ganga Prasad Sen, the Noble being of Veterinary Science of 21st Century, was born on 1stJanuary, 1932 at Vikrampur Bharakar, Dhaka (Now in Bangladesh). He did B.Sc. in 1953 from Calcutta University and B.V.Sc. and A.H. from Bengal Veterinary College, Calcutta in 1958, with Distinction. He did M.V.Sc. (Bacteriology) from Indian Veterinary Research Institute, Mukteswar in 1960 and obtained Ph.D. (in-service) in 1971 both from Agra University.

Dr. Sen started his research career from IVRI Mukteswar Kumaon in 1960 and was appointed as Assistant Research Officer Quality Control in 1961 where he was associated with Draft Preparation of Schedule Fl Central Drugs and Cosmetics Rules, after enactment of which, Division of Standardization was formed at IVRI. From 1965 to 1971 and 1976-78, Dr. Sen served as in-charge FAO/WHO Brucellosis Reference Centre where he was instrumental to the Development of Indian Standards Anti-Brucella abortus Serum and standardized Brucella antigens including Rose Bengal Plate Test Antigen which are still prepared in India as per the same specifications and obtained recognition of Dr. M. Abdussalm, the then Chief Veterinary Public Health, FAO/WHO. He developed New Brucella Phage which is still maintained as International Standard by CVL, Weybridge, UK. By virtue of his services WHO granted Funds to IVRI, Izatnagar, U.P.

He was placed in 1971 at All India Institute of Hygiene and Public Health, Kolkata to teach and guide research in MVPH (course initiated by WHO in collaboration with IVRI and Govt. of India) and Ph.D. of Calcutta University. The Senate and Syndicate of University of Calcutta by special resolution recognized him as University Professor to hold the position of Convener Ph.D. Committee in Veterinary Science. In 1988, he was assigned to establish the IVRI Eastern Regional Station which he succeeded to do at Belgachia, in Kolkata from where he retired on superannuation on December 31, 1991.Dr. Sen developed a School of Thoughts in Veterinary Public Health and was honoured with Fellow of Indian Public Health Association which he served as Honorary Treasurer, General Secretary and Vice-President and represented the World Federation of Public Health Associations in World Health Organization SEARO Committee meetings. In recognition of the specialized services rendered by him Dr. Sen was awarded Golden Jubilee Awards of IPHA by Hon'ble Speaker Sri Somnath Chatterjee and Shri Buddhadeb Bhattacharya. Dr. Sen

was honoured by ICAR as Emeritus Scientist in 1992. He was usually awarded to meritorious top grade renowned scientists in the country.

Dr Sen published more than 130 Research papers, Co-author of two ICAR Monographs, guided more than a dozen of Masters degree and half a dozen Ph.D. scholar including FAQ, CSIR, ICAR and ICMR Fellows. Dr. Sen was President for 1992- 1993 of Medical and Veterinary Sciences section of Indian Science Congress Association.

Dr. Sen had a dream of establishing Veterinary Public Health, a special subject in the Veterinary Education. He succeeded in it and Veterinary Public Health was introduced at Research Level, then at Post Graduate Level, later on at Graduate Level also under VCI norms and standard. This was not so easy, a Herculean Task, which was done by Prof. Sen sacrificing his personal comfort, energy and valuable time for material gains by virtue of his leadership for infusion of the concept of VPH to other Veterinarians, medical and health personnel, social workers and administrators. The "Association of Public Health Veterinarians" was formed by Late Prof. G.P. Sen, as the Founder President; considering all the Veterinarians as public health personnel as they are well versed with Herd Medicine serving the vertebrate animal species for the benefits of human health (community health).

Dr. Sen breathed his last on 29th November, 2010 at Saltlake, Kolkata. We record with profound sorrow the untimely demise of Prof. G.P. Sen. It is an irreparable loss not only to the APHV or Public Health Veterinarians of the country but a great loss to the Veterinary Profession as a whole. The best way to pay homage to him, on this eve will be that we could take Oath to complete his Dreams and put high the "Association of Public Health Veterinarians" as per his wishes.

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The Link between Biodiversity Degradation and Zoonotic Diseases

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Abstract

According to the World Health Organization (1959) "Zoonoses are diseases and infections which are naturally transmitted between vertebrate animals and man". Today, about 60% of all infectious diseases are zoonoses and more than 75% newly emerging infectious diseases originate in animals and 71% of the total events of emergence of infectious diseases originate in wildlife and disease emergence is considered to have a close association with biodiversity. Biodiversity is often understood in terms of the wide variety of plants, animals and microorganisms, the genes they contain and the ecosystem they form- a result of billions of years of evolution. There are increasing researches showing that higher biodiversity markedly reduces the rates of pathogen transmission thereby arresting the numbers of spill-over events lowering the emerging zoonoses risks for human beings. The most studied inhibitory effect of increased biodiversity on pathogen transmission or spill over is known as "dilution effect." Several anthropogenic and so-called developmental activities cause the degradation of biodiversity thereby reducing the dilution effect resulting increased risk of spillover events and disease emergence. To mitigate the occurrence of these dreadful events in future conservation of biodiversity is needed.

Keywords: Biodiversity degradation, Emerging zoonoses, Anthropogenic activities, Zoonotic spillover

Introduction:

Zoonosis is a much-talked subject to the present-day public health experts. It has received more intensive attention once the universe has experienced the horror of COVID 19. Spillover of diseases are not a new thing, rather it has a long past. Recent literatures in relation to the diseases transmitted between vertebrate animals and man (zoonoses), strongly suggest that the number of spillover events are on the rise since the middle of 20th century. A total of 335 events of disease emergence occurred between 1940 to 2004 were studied by a group of scientists led by Jones E. Kate. Their observation revealed that the disease spill over events are highly associated with emergence of zoonotic diseases and about 71% of the total numbers of disease emergence is of wildlife origin and somehow linked to the degradation of biodiversity through different anthropogenic activities (Jones et al., 2008). Researchers from all over the world have also identified thousands of microorganisms representing from viruses, bacteria, and fungi those are originating from animals especially from wildlife are having spillover potential and are spread over almost all continents of the globalized world (Carlson, 2020). Because 60 % of all known infectious diseases of human being are of animal origin and among the newly emerging infectious diseases 75% are of animal origin (Vidal John, 2020), it is high time to be on high alert for setting proactive holistic approach towards the mitigation of this global challenge.

Although different developmental activities at all levels should be put on right track, at the same time it is also important to do it in the context of the recent pandemic. We should not ignore the lessons that we have learnt out of it and consider it as an opportunity to use all our natural resources in a sustainable way for gaining maximum benefits.

Zoonotic Diseases:

Sometime between 1880 to 1885, Rudolph Virchow, A German physician, and pathologist, while working on a pig parasite *Trichinella*, for the first time coined the term 'zoonosis'. He also made a famous remark out of his wisdom that "between animal and human medicine there are no dividing lines-nor should there be." About 75 to 80 years of using this term by Dr. Virchow, the World Health Organization (WHO) in 1959 for the first time defined the term 'Zoonosis'. According to the WHO "Zoonoses are diseases and infections which are naturally transmitted between vertebrate animals and man" (NCDC, 2016). More than 100,000,000 of cases and 1,000,000 of deaths across the world occur every year from zoonoses (Nair, 2020) and the most concerning part is that, in most of the emerging zoonoses we do not have any medicine or vaccine to restrict its spread.

Impact of zoonoses:

The impact of zoonoses could well be realised by the human civilization during the COVID19 pandemic. In fact, the spread of this pandemic has brought the global economy to its knees. COVID-19 has significantly wedged all sectors of the society including health and education sectors, financial sectors, travel and hospitality sectors, and the sports sector (Ozili and Arun, 2020). Travel industry is poised to lose significant revenue due to the pandemic (Ozili and Arun, 2020). The pandemic with its innate nature stifled the human civilization up to every nook and corner by two methods: first, the spread of the virus encouraged social distancing which led to the closure of economic markets, corporate offices, businesses, and events. Second, the augmented rate at which the virus was dispersing, and the amount of uncertainty about how bad the situation could get, led to sudden shift to safety in consumption and investment among consumers, investors and international trade partners. It is estimated that millions of people will face extreme poverty due to the stalled growth resulting from this pandemic (Ozili and Arun, 2020).

Zoonotic diseases or similar kind of emerging infectious diseases hugely affect the travel industry. One of such many examples is COVID 19 pandemic. The International Air Transportation Association (IATA) stated that the air travel industry would lose US$113 billion if the COVID-19 outbreak was not quickly contained. The losses are encountered due to increased flight cancellations, cancelled hotel bookings and cancelled local and international events, imposition of stay-at-home policies etc. It also impacts on sports and similar events. Different mega sport events linked to football, formula one, hockey, rugby, cricket, baseball, lawn tennis, motorsports and many more were either suspended or delayed due to COVID 19 pandemic. The Tokyo Summer Olympic and Paralympic games were also postponed. Different events industry was hit financially by many cancellations — exhibitions, live music shows, conference, weddings, parties, corporate events, brand launches, trade shows, and more. The global film industry incurred a $5 billion loss during the corona virus outbreak. The International Alliance of Theatrical Stage Employees (IATSE) reported that an estimated 120,000 down the stream auxiliary industry jobs were lost due to the pandemic, most of which were theatre-stage employees. The worst affected sector was the education sector. The COVID 19 also disordered the $600 billion higher education business. Persons related to this field all over the world felt the ripple effect of the pandemic as educational institutes were instructed to shut down after the corona virus was declared a public health emergency in many countries. The outbreak had a more severe consequence on schools that did not have an online learning platform (Ozili and Arun, 2020).

Emerging zoonoses and the cross-species transmission (spillover):

Many a times, 'spillover' or cross-species transmission has been found to be an important instrument in the emergence of zoonotic diseases. It can be defined as the "cross-species transmission of a parasite into a host population not previously infected" (Ellwanger and Chies, 2021). To emerge a pathogen through spillover, it needs to overcome two distinct ecological barriers namely **transmission barriers** and **species barriers**. Important routes to overcome the transmission barriers are direct contact, indirect contact, airborne transmission, oral ingestion and through bites of arthropod vectors. When organisms are there within the animals, may be domestic or in wildlife, they form the pathogen pool. And the very few organisms from the pathogen pool who qualify to cross the transmission barriers will form the propagule pool. Now organisms from this propagule pool need to cross the species barrier. Crossing this species barrier is again governed by several factors like phylogenetic distance between the source and the recipient host species, spatial distance naturally maintained between the animal species and human, pathogenic diversity hosted by the animal species etc. Again, the very few negligible numbers of organisms those qualify to cross the species barrier will form the zoonotic pool to spill over to human host resulting a new episode of zoonotic emergence. In the past 100 years, the emergence of zoonotic diseases has accelerated. The 2020 Intergovernmental Platform on Biodiversity and Ecosystem Services (IPBES) workshop report on biodiversity and pandemics reviewed scientific evidence which demonstrated that pandemics are becoming more frequent. They reported that the menace of pandemics is growing rapidly with more than five new diseases evolving in people every year, any one of which has the capacity to spread and become pandemic (Bedenham et al., 2022).

Biodiversity its concept, importance, and threats:

Biodiversity is a wide-ranging umbrella term that denotes the variety of different forms of life on earth, including the different kinds of plants, animals, micro-organisms, the genetic materials they contain and the ecosystem they build up. It is a result of billions of years of evolution. In other words, biodiversity is the food we eat, the water we drink and the air we breathe. More precisely, it is the part of us, as we humans are part of nature. It is assumed that around 52 million species may be there on earth, of which only 2.1 million are known to man (Mora et al., 2011). Biodiversity can be classified as Genetic Diversity (variation of genes within species and populations),

Species Diversity (variety of species or the living organisms) and Ecosystem Diversity (variety of habitats, biotic communities, and ecological processes). Again, Species Diversity is of two kinds namely *Species Richness* (total count of species in a defined area) and *Species Abundance* (relative numbers among species). It provides us with several utilitarian benefits in the form of agricultural materials (all kinds of foods both plant or animal origin), medicines, industrial raw materials; ecosystem services by mitigating climate change, maintaining CO2 and O2 balance, regulating biochemical cycles, removing pollutants and waste materials, ecosystem resilience and also providing ethical and moral benefits and aesthetic values in human life. Following are some of the amazing facts those are reinforcing the undoubted role of biodiversity in our life (Day et al., 2012):

- Some 200 million people depend on wild species for at least part of their food.
- Even in USA, the most technologically advanced country half of the 100 most prescribed drugs originate from wild species.
- 80 % of people live in Africa rely on traditional medicines as main source of their health care need.
- Microbes have given us nearly all of our antibiotics.
- One third of the world's food, worth over US$ 190 billion per year, depends directly or indirectly on pollination carried out by insects, bats, or birds.
- A single colony of Mexican Free-tailed Bat eats more than 9,000 kg of insects per night targeting especially major crop predators.
- A single brood of woodpeckers can eat 8,000 to 12,000 harmful insect pupae per day.
- Frogs provide a range of pharmaceutical compounds starting from pregnancy test kit, antibacterials, antifungals, painkillers and even mosquito repellants attracting the species name "Hopping Pharmacy."

However, species extinction is a natural part of Earth's history but human activity has increased the extinction rate by at least 100 times compared to the natural rate. A square kilometer of coastal ecosystem such as mangroves forests can store up to five times more carbon than the equivalent area of mature tropical forests. But world mangroves are being destroyed 3 to 4 times faster than forests increasing more and more carbon accumulation (IUCN: facts and figures on biodiversity, 2012). As rate of extinction of species is a key indicator of the health of a biosphere, which is again linked to the health of people, the present rates of species extinction, which are 100 times, and in some cases 1,000times or more, faster than those observed in the fossil record is of great concern (Pimm et al., 1995). Biodiversity is declining rapidly due to factors such as habitat alteration and destruction by the land use change, over exploitation of biological resources, climate change, pollution, and invasive species. Such natural or human-induced factors tend to interact and amplify each other (Rawat and Agarwal, 2015). As species extinction events are happening in an unprecedented rate in human history, both researchers as well as policy makers are more and more focused to find out how the variety, distribution, and abundance of life that is biodiversity on a particular landscape are influencing health as well as the zoonotic disease transmission in that area.

Biodiversity loss and zoonotic diseases:

Thousands of infectious agents circulate in human population. Many of them at time circulated in other vertebrate animals where they might have caused diseases to their natural hosts or simply without causing any harm to them. Irrespective, at a certain point they spilled over into human and started to cause diseases. In present time, there is a tendency among the workers to work on zoonotic viruses among the microbes because they are considered as the most likely to cause emerging zoonotic diseases. Initially, there was confusion over the role of biodiversity in pathogen transmission. It was thought that virgin natural areas with high biodiversity were seen as likely sources of new zoonotic pathogens, suggesting that biodiversity could have negative impacts on human health. Later biodiversity has been recognized as potentially benefiting human health by reducing the transmission of some pathogens that have already established human populations themselves in. This apparently opposing effect of biodiversity in human health was due to the different alternative conceptual models used by the researchers linking host diversity to zoonotic emergence in humans. Researchers from the former group used the ***"Total Host Diversity"*** model, where the overall host diversity of a particular area were considered as a source of pathogen pool, any one of which could jump to humans resulting an event of spillover. Whereas, the second alternative model which is also known as ***"Zoonotic Host Diversity"*** model presumed that not all the other vertebrates-rather certain groups- such as bats, rodents, or livestock- might be significantly more likely to act as sources of zoonotic diseases. And in the third alternative model named ***"Zoonotic Host Diversity and Abundance"*** model considered both the diversity and the abundance of the animals most likely to act as hosts for zoonotic pathogens were critical. The last two models were gained more confidence among the researchers when it was also evidenced by different researchers that not all the taxa are equally capable to transmit zoonotic diseases, rather some are more likely to do so. Different research groups have identified different vertebrate taxa as the more likely to transmit pathogens to humans. For example, ungulates

followed by carnivores and bats (Woolhouse and Gowtage-Sequeria, 2005), bats (Dobson et al., 2005), rodents followed by bats, carnivores, ungulates and primates (Johnson et al., 2020) and few of them identified rodents as the group hosting the greatest number of zoonotic viruses (Johnson et al., 2020 and Mollentze and Streicker, 2020). Some of the researchers also proposed domesticated species to act as a "bridge host" for zoonotic pathogens where they transmit the pathogens after acquiring them from the wild hosts (Borremans et al., 2019). But, finally these are the five Orders of mammals namely Primates, Cetartiodactyla, Carnivora, Rodentia and Chiroptera who have been considered as the most common sources for zoonotic pathogens and thereby the "Total Host Diversity" model lost its appropriateness.

Once it was evident through different studies that some of the vertebrates were more likely to act as the source of zoonotic pathogens, researchers started to find out the effects of different anthropogenic activities on those zoonotic hosts. Interestingly it was observed by some scientists that species that host zoonotic pathogens were more likely to be of lower conservation concern that means they were more abundant (Johnson et al., 2020). They suggested that there was a positive correlation between the zoonotic host status and the resilience to anthropogenic activities like change in land use pattern, overexploitation in the form of hunting, trade, pollution, intrusion of invasive species etc.

In one study, the effect of human impacts on host diversity and abundance was analyzed and reported that wild species known to be zoonotic hosts were more abundant and more diverse (as measured by species richness) inhuman-impacted habitats compared to less disturbed habitats. In contrast, wild species not known to be zoonotic hosts declined in diversity and abundance in human-impacted habitats (Gibb et al., 2020). Mendoza et al., 2020 came to a similar conclusion using a smaller dataset of ecological communities and zoonotic hosts.Gibb et al., 2020 also provided evidence that the diversity and abundance of animals in human-impacted habitats shifts toward species that are more likely to be competent zoonotic hosts. In this way, the "zoonotic host diversity and abundance" model became a more realistic than the other two models.

It was further reported by the scientists that zoonotic reservoirs have significantly "faster" life histories – including shorter gestation periods, larger litters, lower neonate body mass and younger age at sexual maturity – compared to non-reservoirs (Plourde et al., 2017; Steams, 1976). To explain this typical association researchers suggested that there is a trade off in investment in innate versus adaptive immunity, with shorter-lived species investing more in the former while longer lived species invest more in the latter (Streicker et al., 2013). That means mammal species that harbor a greater number of pathogen species are more abundant in human impacted habitats and there may be mammalian traits that impact both tolerance to human disturbance and tolerance to infection (Gibb et al., 2020).

It is evident through scientific research that when biodiversity is lost from ecological communities, the species most likely to disappear are large-bodied species with slower life histories (Hutchings et al., 2012), while smaller-bodied species with fast life histories tend to increase in abundance (Keesing and Young, 2014). Recent research shows that fast-lived species are more likely to transmit zoonotic pathogens (Plourde et al., 2017). Together, these processes are likely to lead to increases in the abundance of zoonotic reservoirs when biodiversity is lost from ecological systems. Taking deforestation or fragmentation of forest as one of many important anthropogenic activities that causes the loss of biodiversity the above situation can be well explained. The undisturbed intact forest ecology can well support all the habitats in their own areas, i.e., the larger sized animals into the deep core forests and smaller in the periphery which is called the edge of the forests. Now, fragmentation of forest will reduce the area of core forest and at the same time increase the edge area of the forests resulting in the decline of the numbers of large-bodied species and increasing the numbers of smaller-bodied mammalians ultimately declining the biodiversity. This is called "edge effect." And as because of fast-lived smaller – bodied species are more likely to transmit zoonotic pathogens, there is increased possibility of occurrence of spill over events. At the same time, in a more diversified ecosystem, the same sized pathogen pool had a much wider scope to be diluted ('dilution effect') among the both efficient as well as the inefficient hosts rather than a less diversified efficient host ecosystem resulting in a better disease transmission scenario. Therefore, any anthropogenic activities like deforestation, intensive agriculture, fossil fuel extraction, mining, and other types of land use changes that causes biodiversity losses ultimately causes the loss or reduction of dilution effect increasing the risks of spillover events and zoonotic disease emergence.

Conclusion:

Following strategies may be prioritized for mitigation of the zoonotic spillovers in relation to the biodiversity degradation-

1. First, we should recognize zoonoses as a problem through understanding the health as well as socio economic consequences of these diseases.

2. We should also understand that the degradation of biodiversity across the globe is the main factor associated with the emergence of zoonotic diseases.
3. We should find out the diverse communities of host species serving as a source of new pathogens.
4. As viruses have been recognized as the most likely pathogens to cause disease emergence, detection, and identification of potential zoonotic viral threats to human health and food security is to be done.
5. Determination of geographic distribution and ecologic scopes of those viruses to inform risk and surveillance in human and animals is the need of the hour.
6. Monitoring of the movement of detected viruses across hosts and regions is to be done.
7. Improving the assessment of the risk of spillover and epidemic potential and forecasting to prevent the possible epidemic.
8. Conservation of biodiversity by using different in-situ as well as ex-situ conservation methods.

Conflict of interest:

The authors declare that no conflict of interest exists.

Author's contribution:

CD prepared the manuscript and RaB and RB critically reviewed the same.

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Bovine Tuberculosis in India: The Need for One Health Approach and The Way Forward

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Abstract

Tuberculosis is one of the most common highly infectious bacterial zoonotic disease, causing significant morbidity and mortality worldwide, particularly in developing countries. *Mycobacterium bovis,* a member of the *M. tuberculosis* complex, has socio-economic importance or public health implications, and may have a sizable effect on the commerce of animals and animal products internationally. It is also the cause of zoonotic tuberculosis. TB occurs in every part of the world. It is one of the top 10 causes of death worldwide than the leading cause of death from a single infectious agent (Ranked over HIV/AIDS). The World Health Organization (WHO) has implemented a new strategy for TB prevention, treatment and control since 2015- The End TB Strategy aims at ending the global TB epidemic by reducing the incidence and death frequency of the disease by 90% and 95% respectively in 20 years (2015-2035) compared to 2015 through advanced diagnosis, prevention and control strategies.

Keywords: Bovine tuberculosis in India, Zoonotic potential, Control programme, One Health approach

Introduction:

Bovine tuberculosis is a chronic granulomatous inflammatory disease that is predominantly caused by *Mycobacterium bovis* (Cousins, 2001). In case of bovines, the disease is insidious in nature and may affect the body tissue, but the lesions are frequently observed in the lymph nodes (particularly of the head and thorax), lungs, intestines, liver, spleen, pleura and peritoneum. Studies have shown that wildlife and domestic animals share common *M. bovis* genotypes suggesting transmission of organism between these animal species (Naranjo et al., 2008).

Bovine tuberculosis affects a wide range of domestic vertebrate animals. Human, cattle, deer, pigs, domestic cat, wild carnivores, and omnivores are among the mammalian host of bovine tuberculosis. Human tuberculosis due to *M. bovis* is one of the main reasons for interest in bovine tuberculosis.

Mycobacterium tuberculosis complex (MTBC):

Mycobacterium tuberculosis complex (MTBC) is closely related group of bacteria that includes *M. bovis, M. bovis BCG, M. tuberculosis, M. africanum, M. microti, M. orygis, M. canetti, M. pinnipedii* and *M. caparae* (Jagielski et al., 2016). It is observed that the members of the MTBC group share 99% genetic similarities at the nucleotide level, confirmed by High-performance liquid chromatography (HPLC) patterns, 16S rRNA and hsp65 gene sequences (Brosch et al., 2000). Despite their close genetic relatedness, they differ in human tuberculosis disease epidemiology, pathogenicity, geographical distribution, host affinity, and severity. *M. orygis* is most likely a previously unknown Indian bovine pathogen becoming more relevant as a global pathogen (Van Ingen et al., 2012).

Zoonotic potential of TB:

Zoonotic tuberculosis (TB) is a chronic, progressive, and infectious illness mostly caused by *M. bovis,* which has a high tendency to infect people. Only a very small proportion of Tuberculosis in humans is caused by *M. bovis*. The most important method of transmission of zoonotic TB is through the consumption of contaminated raw milk and milk products such as butter, dahi (curd), milk cream, cheese, and improperly cooked meat or raw meat.

Reverse Zoonosis:

Hence the pathogen can be transmitted from human to non-human, in several investigations from Africa and India, incidence of reverse zoonosis had been found in Africa and India. Particularly, the animals serve as a vehicle of transmission for drug-resistant *M. tuberculosis* due to reverse zoonosis at the human-animal interface in areas where poor animal management and meat inspection are combined with a high burden of MDR-TB. Infection of *M. tuberculosis* has been observed in many domestic and wild animal species, with most cases occurring in animals that have spent an extended period near people. The *M. tuberculosis* infection has been found more in cattle than other livestock animals. *M. tuberculosis* is more prevalent in Indian cattle than *M. bovis.*

Status of animal Tuberculosis in the world:

The information on the global distribution of bovine TB (bTB) is limited, but the disease has been reported from all the livestock producing middle and low income

countries of the world. Tuberculosis is now the leading infectious disease cause of death in the world. TB is now the single disease that causes the greatest mortality in the world, at over 1.6 million deaths each year.

Bovine tuberculosis in India:

There are 303.76 million bovines in India, and it is the largest producer of milk and the second largest producer of meat worldwide (NDDB, 2021). The prevalence of bTB among farm and dairy cattle in India is estimated to be around 7.3%, that means around 21.8 million cattle are estimated to be infected in India which exceeds the entire population of dairy cows in the United States; which makes it a country with one of the largest infected herds in the world (Srinivasan et al., 2018). While bTB control programs have had considerable success in reducing the prevalence of the disease in many developed countries, they have yet to be formulated or implemented in India. India alone accounted for 26% of the TB prevalence and 34% of TB deaths globally in humans in 2020, the highest burden globally (WHO, 2021). Bovine TB also has a zoonotic and reverse component, which means that the disease can spread from cattle to humans and from human to cattle. The formidable "End TB Strategy" by the World Health Organization (WHO) and the National Tuberculosis Elimination Program (NTEP) by the Government of India will be a great success only when all routes of transmission, including zoonosis and reverse zoonosis are taken into consideration (Figure 1).

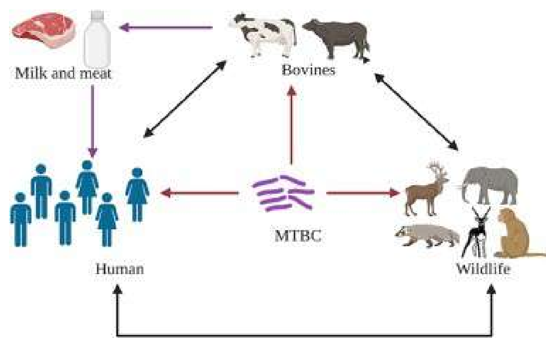

Figure 1: The transmission chain of TB between humans and animals

Bovine tuberculosis control programs across the globe:

The incidence of bTB has been fairly restricted in developed countries with systematic bTB eradication programs, but successful eradication and maintenance of bTB-free status are still significant challenges. In addition to threatening public health, bTB is also a significant economic concern, estimated to cost $3 billion worldwide annually, accounting for losses from reduced cattle productivity, culling, and movement and trade restrictions. In most developed countries, bTB control programs have existed for a long time, where 'test and cull' strategy is a major approach. This resulted in exceptional benefits to human health while fetching nearly a 10-fold return on investment in animal rearing and productivity (Waters et al., 2012).

In 1917, the 'test and slaughter' policy was introduced in the USA. By 1940, 232 million TB tests were administered in cattle resulting in the culling of 3.8 million cattle throughout the country. This approach, coupled with mandatory milk pasteurization, led to the prevention of 25,000 TB deaths in humans in 1940, a period when chemotherapy was not readily available for the disease. Presently, in the USA, cattle herds' surveillance is undertaken using ante-mortem and post-mortem methods. The United States Agricultural Department (USDA) oversees the status of bTB in the country using the methods described in the 'Uniform Methods and Rules' that are in effect since 2005.

Similarly, in Europe, skin testing and interferon-gamma assay are used to test herds for awarding Officially TB Free (OTF) status to herds with no reactor cattle. However, the presence of one reactor animal leads to OTF- Withdrawn (OTFW) status, leading to quarantine and slaughter of the herd. Post-mortem examinations of slaughtered cattle are also often carried out in these countries by trained veterinarians (Lawes, 2016). Slaughterhouse surveillance is a critical component of the bTB eradication and surveillance program.

Need for bTB control policies in India- the human front:

Bovine TB has been reported in India since 1917. Since then, the disease has become endemic in India and sporadic in the West (Mallick and Aggarwal, 1942). The prevalence of TB in humans in India is 3120 per million population for the year 2021 as per the National Tuberculosis Prevalence Survey. According to the Global Tuberculosis Report 2021, about 26% of the TB cases reported worldwide in 2020 were from India. In a country with such a high TB burden in humans, it is imperative to curb the spread of the TB disease as soon as possible.

For a long time, it was surmised that *M. tuberculosis* is the etiological agent for TB infection in humans, and *M. bovis* is the etiological agent for TB in animals. According to the WHO Global TB report, zoonotic TB is defined as a disease caused by *M. bovis* in humans, and its mortality is 2020 in the South East Asian region alone (WHO, 2020). However, recent discoveries have challenged this notion making it necessary to reassess the cause and effect of bTB in humans and animals. The isolation of *M. orygis* strain in comparative intradermal test (CIT) - positive cattle from a farm in Chennai using whole genome sequencing was reported in 2019 (Refaya et al., 2019). A cross-sectional surveillance study

done in three farms in Chennai to test for tuberculosis among cattle and animal handlers revealed those four cattle and six animal handlers were infected with the same strain of *M. tuberculosis* (Palaniyandi et al., 2019). In 2020, a study conducted at the Christian Medical College in Vellore, South India, reported the isolation of 7 *M. orygis* strains from humans using culture, molecular methods, and whole genome sequencing (Duffy et al., 2020). *Mycobacterium orygis* has been included in the MTBC since 2012 as a separate sub species that was initially known to cause infection in oryxes. The isolation of *M. orygis* from cattle and the identification of the same species in humans in India have raised concerns about the broad host spectrum of the pathogen.

On the human front, TB caused due to *M. bovis* is treated the same way as TB by *M. tuberculosis*. The NTEP in India does not delineate the MTBC in human TB infection, and hence does not report the causative organism for individual TB cases. This has masked the true prevalence of TB caused by individual members of the MTBC. Moreover, the same treatment regimen of isoniazid, rifampicin, pyrazinamide, and ethambutol is advised for all patients diagnosed with TB (RNTCP, 2017). While *M. tuberculosis* is susceptible to all the drugs, *M. bovis* is not susceptible to pyrazinamide.

Animal movements as a factor of bTB spread:
Testing of live animals that are exported and imported for the dairy industry, meat, etc. could prevent the transmission of bTB and hamper the introduction of new strains in the population. In the Indian context, cattle are often exported to Bangladesh and imported from Bhutan illegally due to porous international borders. Cattle are exported to Bangladesh from West Bengal and Tripura due to the rising demand for beef and other by-products like leather. Since slaughtering live animals for meat is a taboo in Bhutan, animals are imported to Arunachal Pradesh and Assam borders in India, where animals are slaughtered and cleaned. The meat is then supplied to markets across the border to Bhutan, where it is sold for human consumption.

Tuberculosis in wild animals as a threat to bTB eradication:
A significant hurdle many countries face in eradicating bTB is the presence of wildlife hosts. Bovine tuberculosis has a range of hosts, making the risk of spillback infections from wildlife to cattle and/or humans and establishing wildlife reservoirs more prominent. Such wildlife reservoirs have been widely reported across the globe. Such wildlife reservoirs make bTB control measures moot, posing the risk of spillback infections to other hosts. In India, *M. tuberculosis* has been identified in wild elephants post-mortem confirmed by culture and PCR methods (Zachariah et al., 2017). *M. tuberculosis* has also been similarly identified from one gazelle. *M. orygis* was isolated from free-ranging spotted deer and black buck in Guindy National Park in India (Refaya et al., 2022).

Presence of MTBC organisms in the environment:
The environment as a source of TB transmission has yet to be considered. Since MTBC bacteria remain in the environment long enough to pose a risk of exposure to many species cohabiting in the same habitat, it could potentially promote indirect transmission (Ghodbane et al., 2014). Some studies have shown isolation of MTBC species from soil and water samples in TB endemic regions.

Major needs and recommendations – towards a One Health approach:
The prevalence of bTB infection in cattle and other hosts and associated risk factors have not been characterized sufficiently in India. This gap in literature needs to be addressed, and initial prevalence reports of bTB and zoonotic TB (zTB) have to be wrought. Preventing the transmission of infection from bovine to humans and vice versa is the primary need. A comprehensive One Health approach could be a significant foot forward. An effective One Health program requires strong political will, evidence-based policy innovations, clearly defined agency duties and tasks, coordination mechanisms at all levels, and an open information exchange culture. Coordination between the veterinary, clinical, epidemiological, and public health sectors is necessary to design and structure TB eradication programs that integrate both the human and fronts. This would lead to better One Health practices that will help eradicate TB. Timely reports of infected herds and immediate steps to prevent transmission could be achieved through amicable and synergistic actions taken by these sectors. Some recommendations for the eradication of bTB are as follows.

1. Raising awareness among animal handlers:
Animal handlers come into direct contact with infected animals; as a result, it is imperative that they be informed about bTB and the effects it can have. The diagnosis of bTB depends upon the animal handlers' ability to identify the disease's initial symptoms. This would enable them to seek help from healthcare and veterinary service providers in both the public and private sectors. This would also help collect data regarding the diseased animals. Animal handlers and people in associated professions should be made aware that they are stakeholders in eliminating bTB and their crucial role in the process.

2. Development of human resources:
The elimination of bTB requires well-trained human resources in public health. This initiative illustrates a

significant transdisciplinary One Health initiative that integrates zoos and public health organizations (Robinette et al., 2017). Such initiatives could be undertaken in India regarding bTB, where the spectrum of healthcare workers could be trained in the prevention, diagnosis, and treatment of both TB and bTB.

3. Ante-mortem and post-mortem diagnosis of bTB in cattle:

As described earlier, ante-mortem testing of cattle for bTB has played a significant role in bTB eradication programs worldwide. Once the animals are diagnosed as positive reactors, they must be segregated from the herd. This prevents the spread of disease within the animals in the respective herds. Ante-mortem testing can also be used to identify humans who are at risk of TB disease. In the initial stages of the program, cattle owners can also be given incentives for maintaining bTB-free herds to motivate them to participate in the eradication.

Routine slaughterhouse surveillance in abattoirs in India is not a norm. Slaughterhouse surveillance can play a pivotal role in bTB control, especially in endemic areas, through proper meat inspection protocols.

4. Mandatory pasteurization of milk:

Nearly 46% of the milk produced in India is either consumed by the producers, most often in the unpasteurized form, or sold to non-producers in rural areas. In contrast, the remaining 54% is available for sale to organized and unorganized sectors. The unorganized/informal sector involves local milkmen, contractors, etc. (DAHD, GOI, 2019). Several studies from India have documented the isolation of either *M. tuberculosis* or *M. bovis* from milk samples collected from infected animals. Stringent laws regarding pasteurization of milk in India could significantly lower of the prevalence of the disease.

5. Contact tracing of infected cattle:

In India, about 80 million rural households engage themselves in the production of milk. A large proportion of milk producers (about 95%) in the country hold 1 to 5 milch animals per household as a part of the subsistence farming system (DAHD, GOI, 2019). This means a significant population of the country share living quarters with animals and is in close contact with them. This results in a greater zoonotic and reverse zoonotic risk. Contact tracing of humans and cattle in close proximity to the infected animals will help identify diseased herds and humans to begin preventive steps to deter transmission at the earliest.

6. Vaccination of cattle:

Vaccination of cattle and other reservoirs of bTB is hypothetically the cornerstone of a bTB eradication program. The BCG vaccine has been used in countries like the United Kingdom, USA, Africa, Canada, and Australia but with no apparent success. A systematic review and meta-analysis by Srinivasan et al., in 2021 reports an overall vaccine efficacy of 25%. The study further speculates that in high disease-burden countries (prevalence between 20 and 40%), 50–95% of cumulative cases may be averted over the next 50 years, and in the case of low to moderate (prevalence <15%) settings, officially TB free status can be achieved in the next ten years upon immediate implementation of BCG vaccine.

However, this strategy suffers a distinct disadvantage- the test sensitizes cattle to tuberculin skin tests. If the test and slaughter policy is administered in India, a positive reaction to the tuberculin skin test will result in culling productive disease-free cattle. Recently, vaccine research has focused on antigens that do not react with tuberculin skin tests i.e., those antigens that are absent in BCG but present in *M. bovis*. The ESAT-6, CFP10, and Rv3615c antigens have shown a possibility for prospects in this area (Vordermeier et al., 2016). In addition, other developments, including the innovation of DIVA (differentiate diseased animals from vaccinated animals) tests, might enable the BCG vaccine to be a part of routine testing as well.

Conclusion:

The above discussion has presented information about the current state of bTB in India, where the importance and necessity of bTB surveillance programs is increasing at the human-animal-environment front. It is imperative to form new policies that would systematically eradicate the disease in humans, animals and the environment. Incorporating the One Health approach to attain this goal will ensure the eradication of origin and disease transmission from all fronts.

Conflict of interest:

The author declares that no conflict of interest exists.

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One Health Perspective on Antimicrobial Resistance

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Abstract

One Health is the collective efforts of multiple health sciences to achieve optimal and best health for people, domestic animals, wildlife, plants, and our environment. The antimicrobial resistance originates from the abuse of antimicrobials in human, animal, and environmental sectors and the spread of antibiotic-resistant bacteria and resistance determinants within and between these pathogens/commensals sectors around the globe. Most of the classes of antimicrobials used to treat bacterial infections in humans are also used in animals. That makes the issue more challenging. Anti-microbial resistance (AMR) is an international issue nowadays affecting not only human health but also animal health as well as the environment. In order to frame it under one health concept we need to look at it from different perspective. Colistin (plasmid-mediated *mcr-1* gene), third-generation cephalosporins (Amp-C beta-lactamases) and Quinolones (The *qnr* gene)are key factors of AMR issue. Major concerns in the animal health and agriculture sectors are mass medication of animals with antimicrobials that are critically important for humans, such as third-generation cephalosporins and fluoroquinolones, and the long-term, in-feed use of medically important antimicrobials, such as colistin, tetracyclines, and macrolides, for growth promotion, the emergence and transmission of plasmid-mediated "*big five*" carbapenemase genes. Supporting a "One Health" strategy is crucial for battling AMR (human, animal, plant, and environmental health). This calls for bolstering global governance and accountability, investing in sustainable response, partnering for more effective action, innovating to protect the future, and speeding global development.

Keywords: Antimicrobials, Antimicrobial resistance, One health strategy

Introduction:

In different global health problems, antimicrobial resistance (AMR) is the one of best examples of the One Health approach [WHO, 2014]. There is a greater ability to proliferate bacteria in animals, humans, and the environment when they acquire resistance to any antimicrobial or antibiotic (Collignon et al., 2018). Because of AMR, there are 700000 deaths per annum from AMR in which 90% occur in LMICs (Low- and Middle-Income Countries), according to this in 2030 there is AM use will increase by 67% mainly in LMICs and livestock production will fall in LMICs by 10% and estimated costs world USD 3.4 trillion a year which is equivalent of 40% of global expenditure on health today and 24 million more people forced into extreme poverty. In 2050 there will be 10 million annual deaths from AMR worldwide. Antimicrobial resistance (AMR) is one of the top ten global health priorities, a major cause of death worldwide, and a development danger (FAO et al., 2022-2026).

Antibiotic resistance genes pose a threat of emergence and rapid geographic dispersion, as demonstrated by two recent occurrences. An enzyme that provides defence against a variety of antibiotics, New Delhi metallo-beta-lactamase (NDM-1) has originated in the subcontinent of India and expanded to the UK as a result of medical travel following initial discovery back in 2008 (Yong et al., 2009). Recently, this gene NDM-1 was discovered in Arctic soil rich in nutrients of a remote archipelago in Norway, increasing the likelihood of its spread by the droppings of migratory avian species (McCann et al., 2019). The MCR-1 (mobilized colistin resistance-1) gene, a plasmid-borne gene conferring colistin resistance, was identified in pigs in China in 2014 (Liu et al., 2016) and cases found in other countries (Marston et al., 2016; Wang et al., 2018).

In clinical and natural settings, the overuse or misuse of antibiotics creates an artificial selective pressure that is harmful to both animal and human health (Holmes et al., 2016). It is a natural process for bacteria to evolve resistance. Genetic mutations or the exchange of genetic material through additional resistance genes are the two ways in which resistance develops (Davies et al., 2010).

It is commonly recognized that antibiotics can increase a pathogen's resistance mechanism by horizontal gene transfer. As of right now, antibiotic resistance to carbapenems, polymyxins, and 3rd generation of cephalosporins is quickly growing (Rizzo et al., 2019). The adoption of extended-spectrum β-lactamase (ESBL) genes is linked to resistance in bacterial pathogens

towards third-generation cephalosporins. An estimate states that ESBL-producing gut bacteria are present in about 14% of the world's population and are expanding at a rate of 5.4% per year (Karanika et al., 2016).WHO reported resistance to carbapenems, vancomycin, third-generation cephalosporins, and clindamycin in 2017. The WHO has been spurred by rising germ resistance to antibiotics used as a last resort to provide funding for additional studies aimed at creating innovative antibiotic-lead compounds to combat priority infections (WHO, 2017).

It can be used to comprehend *Escherichia coli* (*E. coli*) due of its high transmission rates among humans, animals, and environmental interfaces. Since it is arguably the most researched, we concentrate on AMRs role in *E. coli* indicator organisms, making it easier to track its spread among animal hosts by anaerobes (Anderson et al., 2006). Additionally, compared to other gut bacteria (such as Bacteroides), *E. coli* may mobilize AMR genes more readily and can survive and even develop outside of the host (Manna et al., 2006). Numerous *E. coli* strains have gained the virulence genes required to produce a broad range of intestinal and other than intestinal diseases, including UTI, bacteraemia acquired in hospitals and communities, and diarrhoea. AMR has increased quickly in LMICs because of a combination of high rates of infectious illnesses, poverty, and population density with lax antibiotic usage regulations and enforcement. Antimicrobial-resistant neonatal illnesses are thought to cause 60,000 newborn deaths annually in India (Laxminarayan et al., 2013).

Since the community is most likely the source of many of these infections, detecting, preventing, and controlling AMR will necessitate a multimodal One Health approach. The existence of resistance to drugs Community-acquired illnesses are because of a variety of interconnected factors, such as the excessive use of antibiotics inappropriately, their accessibility on the open market, in the production of food animals, and the existing level of contamination in both drinkable and ocean surface waters (Skariyachan et al., 2015). As a result, antimicrobial resistance is a prime example of a One Health issue since resistant microbes can spread among animals, humans, and the environment.

One Health (OH) Approach's Perspective on Antimicrobial Resistance:

The quick spread of multidrug-resistant bacteria which cause infections that are resistant to conventional antibiotic treatments across the world is a serious cause for concern. Of the 32 antimicrobials under hospital development listed by the WHO in 2019, just six were considered novel. The world's health systems are being impacted by the shortage of antibiotics. Antimicrobial-resistant microorganism infections are becoming harder to treat because antimicrobials are becoming less efficient at treating them, which raises the death rate. To combat diseases brought on by the main pathogens listed by the WHO, newly upgraded antimicrobials are required. Now, these new (upgraded) antimicrobials will meet the same fate as the current (present) antimicrobials and become useless unless the manner the current antimicrobials are utilized has not altered.

Antimicrobial hold-out because AMR causes lengthy hospital stays with high expenses, it impairs patient or caregiver productivity and has a considerable influence on national economies and health systems.AMR is principally brought on by the misuse and overuse of antibiotics, insufficient hospital infection prevention and control procedures, insufficient access to clean water, hygienic circumstances, and animal and human cleanliness, ignorance, and anomalies in the legal system. In response to the global public health concern of antimicrobial resistance (AMR), epidemiological surveillance systems have been established with the purpose of promoting collaboration targeted at preserving both human and animal health as well as the balance of the ecosystem. The Food and Agriculture Organization of the United Nations (FAO), the World Organization for Animal Health (OIE), and other international organizations have established a Global Action Plan on Antimicrobial Resistance-WHO.

This plan's actions included learning about AMR through research and surveillance. The advisory panel developed guidelines for AMR surveillance to ensure that all countries carry out integrated surveillance that will cover the use and consumption of antibiotics in both human and animal populations. Readers will have a thorough understanding of how AMR spreads in diverse contexts and geographical areas thanks to these guidelines. It will enable research on the relationship between antibiotic use and antimicrobial resistance (AMR) in a range of contexts, including people, animals, and the environment, as well as the assessment of the effects of interventions within and between sectors (WHO, 2017).

The Interagency Coordination Group on Antimicrobial Resistance (FAO, WHO, and OIE) sent a paper titled "We can't: securing the future against drug-resistant infections" to the UN Secretary-General in April 2019. Additionally, a joint tripartite secretariat (FAO, OIE, and WHO) was established with WHO serving as its headquarters to promote multi-stakeholder engagement on AMR. The decision was made to create new governance organizations, such as the global AMR leaders' group, the independent AMR action reporting group, and the multilateral collaboration platform. One of the strategies to raise public awareness of the AMR issue

was the creation of "Global Antimicrobial Awareness Week". It will be named as "World Antimicrobial Awareness Week " beginning in 2020, and it will relate to all antimicrobials, including antibiotics, antifungals, antiparasitic, and antivirals. By raising public awareness of antimicrobial resistance (AMR) and encouraging best practices among the public, healthcare professionals, and policymakers, this global initiative aims to halt the creation and spread of drug-resistant infections. One of the strategies to raise public awareness of the AMR issue was the creation of "Global Antimicrobial Awareness Week". It will be named as "World Antimicrobial Awareness Week" beginning in 2020, and it will relate to all antimicrobials, including antibiotics, antifungals, antiparasitic, and antivirals. By raising public awareness of antimicrobial resistance (AMR) and encouraging best practices among the public, healthcare professionals, and policymakers, this global initiative aims to halt the creation and spread of drug-resistant infections.

WHO launched the Global Antimicrobial Resistance Surveillance System (GLASS) in 2015 with the goal of bridging knowledge gaps and guiding strategies universally. Antibiotic consumption was monitored, surveillance data on human antibiotic use was eventually incorporated, and the impact of AMR in the ecosystem and food chain was understood through the development of GLASS. It provides a standardized approach to collecting, assessing, analyzing, and sharing data across national, regional, and local boundaries. It also shows the correctness and representativeness of the data collected and allows you to monitor the evolution of new or existing national surveillance systems.

In 2017, the WHO created a list of priority pathogens to direct the creation of novel drugs, diagnostic instruments, and vaccines. WHO assesses clinical and preclinical antibiotic development programs annually to determine how well they work against high-priority infections. This was a revised list in 2022. Additionally, the Global Alliance for Antibiotic Research and Development was established through cooperation between WHO and the Drugs for those diseases which the Neglected Initiative collaborated to launch. This alliance funds research and development through public-private partnerships. The partnership aims to develop and implement five innovative treatments by 2025 to tackle drug-resistant bacteria, which the WHO has identified as the greatest threat(Global action plan on AMR Geneva: WHO, 2015).

Use of antibiotics by humans, animals, and plants:

Resistance to certain antimicrobials took decades to emerge, but resistance to other antimicrobials happened considerably more quickly. Vancomycin in particular, an antibiotic with a delayed rate of resistance development, was very costly for its ongoing capacity to treat infections that other antimicrobials were unable to cure. Vancomycin-resistant enterococci, for example, were supposed to be a relatively low health risk. However, they now significantly increase mortality and morbidity, especially in hospitals, making the growing vancomycin resistance a concern (Collignon et al., 2019). Antimicrobials are used in many animal applications, including as farm animals, bees, pets, and farmed fish in aquaculture systems. Antimicrobials are essential to animal husbandry and are utilized for a variety of objectives, including preventive, therapeutic, and development boosters. Worldwide, antimicrobial usage in animals is thought to be higher than in humans. A maximum of human-use antibiotic classes, including those essential to human health care like quinolones and broad-spectrum beta-lactams, are also preferred for use in animals(Jans et al., 2018).

Certain antimicrobials (such as streptomycin, triazoles, and tetracycline) that are prescribed for use in humans and animals are also employed in plants. Resistant bacteria which are zoonotic in nature are also found in the soil infecting vegetation.AMR is easily transported between and between different ecosystems and populations. Antibiotic-resistant fungi are known to be created when in the agriculture sector antimicrobials are used and are then transferred between people from our surroundings (www.fao.org/antimicrobial-resistance). Certain antimicrobial classes—such as carbapenems — are exclusively used in human,while others: such as flavophospholipol and ionophores — are meant only for animals (http://www.fao.org/3/a-i6209e.pdf). Streptomycin and Tetracycline are other clinically useful antibiotics used in treating and preventing fruit infection-causing bacteria (Sundin et al., 2018). It is possible that aquaculture uses higher doses of antibiotics than what is recommended for cattle. Antimicrobial residues can persist in fish products and aquaculture for prolonged time intervals through excretion. These leftovers create selection pressure by spreading quickly in bodies of water (Watts et al., 2017).

Antimicrobials used in the One Health Model:

Fluoroquinolones: Effective for a great range of Gram-positive and Gram-negative bacteria, fluoroquinolones are a class of broad-spectrum drugs used to treat urinary tract and respiratory infections. Fluoroquinolone resistance can be brought on by loss of porins, the existence of efflux pumps, or modifications to topoisomerase IV and DNA gyrase target sites. In 1998, resistance to quinolones administered horizontally was initially reported. Such resistance is due to the q*nr* gene, which is found in a genetic element (Hooper et al., 2015).

Given the significance of fluoroquinolones as antimicrobials for treating human diseases, the preferred

use of these drugs in wild and domestic animals has been designated as an important area. Fluoroquinolone use in Europe reached over 216 tons in 2017, mainly inmeat animals for consumption (EMA/294674/2019). In the USA, fluoroquinolone (about 23.3 tons) usage in animals in 2018, accounting for 2.4% of all antibiotics used in animals in Europe (FDA, 2018).

Colistin: The highly bactericidal antibiotic colistin (polymyxin B) has been used for many years by both humans and animals; nonetheless, systemic administration of the medicine causes nephrotoxicity (Falagas et al., 2006). This antibiotic is limited to usage in the treatment of skin infections or cystic fibrosis patients. However, the frequency of systemic colistin treatment has increased (Wi YM et al., 2017) to treat infections caused by bacteria resistant to carbapenems (*Escherichia coli* and *Pseudomonas aeruginosa*). Colistin is used to treat infections and stimulate animal development in animal husbandry; however, the use of this antibiotic in excess of human dosages has been observed in certain countries (European Medicines Agency, 2019; European Surveillance of Veterinary Antimicrobial Consumption). The plasmid-mediated *mcr-1* gene was found to be the cause of colistin resistance in *E. coli* strains recovered from food samples, animal samples, and blood cultures in China in 2015. It was previously thought that colistin resistance was chromosomally encoded. *P. aeruginosa*, *Enterobacter* spp., and *Klebsiella pneumoniae* are among the other bacterial genera that carry the mcr-1 gene. Other studies have reported that the *mcr-1* gene is present in bacteria isolated from animal, environmental, and surface water samples worldwide (Wang et al., 2018).The evolution of resistance of colistins suggests that using high dosages of antibiotics to treat illnesses or as a growth enhancer may further increase resistance. Using avoparcin as a growth enhancer resulted in the same issue. Furthermore, resistance to serious infections brought on by enterococci has developed because of vancomycin utilized in methicillin-resistant *Staphylococcus aureus* (Bager et al., 1997).

Carbapenems: The antibiotics known as carbapenems are used in treating severe Gram-negative bacterial infections in people. The enormous morbidity, death, and medical costs associated with carbapenem resistance make it a major public health disaster, particularly in developing nations like India. Growing the problem is thespread of the "big five" plasmid-mediated carbapenemase genes in Gram-negative bacteria: KPC, NDM, IMP, VIM, and OXA-48 type. Antibiotic resistance determinants are co-harbored by carbapenemase-producing carbapenem-resistant organisms (CP-CRO), which leads to multi- or pan-drug resistance. CROs are related to humans, animals, and environmental settings. Cross-border transmission of these superbugs is heightened when spills occur in food chains, jeopardizing food safety and security. Across the worldmetallo-β-lactamases in human, animal, and environmental primarily those that produce NDM-1-producing CROs, are frequently exchanged (Madec et al., 2017).

Cephalosporins (third generation): These beta-lactam antibiotics are widely used by both humans as well as animals; cephalosporins, like ceftriaxone and cefotaxime, are utilized to treat a range of human infections, infections of the abdomen, circulatory system, lungs, and the urinary system (Temkin et al., 2018). Veterinarian cephalosporins that are most frequently used are ceftiofur, cefoperazone, cefovecin, and cefpodoxime. Ceftiofur has been licensed by numerous countries to treat bacterial infections, primarily in animals meant for human consumption. Animals may only get it parenterally, either individually or in groups. Depending on the animal type, ceftiofur is used to treat a wide range of illnesses, such as meningitis, septicemia, pneumonia, septic arthritis, polyserositis, and metritis. It is also sometimes used to treat respiratory disorders, prophylaxis in cattle raised for meat production (beef), and prevention of *Escherichia coli* infections in broiler chickens (Gibbons et al., 2016).

Data on antibiotic use collected in Europe, where data has been available for a long period, show that over 18 tons of 3rdand 4thgeneration cephalosporins were used in 2017, predominantly in animals intended for human consumption (EMA/294674/2019). This made up roughly 0.2% of all antibiotic applications for animals in Europe. Approximately 31.44 tons of cephalosporin were used for animal use in the United States in 2018, according to the Food and Drug Administration (FDA).

Extended-spectrum beta-lactamases have the ability to inactivate aztreonam and third-generation cephalosporins, such as ceftriaxone, cefotaxime, and ceftazidime. The genes that encode this resistance are spread via plasmids and transposons. The occurrence of horizontal transfer of genes among enterobacteria was proven by the identification of AmpC beta-lactamases in genomes and their subsequent expression in plasmids (Madec et al., 2017). Serious infections caused by *K. pneumoniae* and *E. coli*-associated cephalosporin strains are now known to be resistant in many different nations. As a result, more people are using certain antibiotics that are currently on the market, like carbapenems (Mughini-Gras et al., 2019). Ceftiofur is primarily used to treat *E. coli* and yolk sac infections. *Salmonella* spp. are the bacteria connected to contaminated poultry products and are known to cause serious illnesses in humans. Research has shown that this therapy selectively selects variants of *Salmonella* resistance to cephalosporins (Saraiva et al., 2018).

Consequences on the health of the public and animals:

Scientific studies have revealed that the careless use of antimicrobials in the veterinary field has led to the development of resistant human-infecting bacteria, including strains of *Salmonella* spp., *E. coli*, *Enterococcus*, and *Campylobacter* (Nelson et al., 2007).

Strains of Salmonellae that became exposed to fluoroquinolones when raised as food are linked to quinolone resistance. Regular administration of ceftiofur has resulted in the observation of resistance to carbapenemase in *Salmonella* strains (Cui et al., 2019).

One significant pathogen that frequently causes bacterial infections, including enteritis, urinary tract infections, and bloodstream infections, is *E. coli*. AMR, or antimicrobial resistance, is a fast-expanding issue linked to *E. coli* infections in both humans and as well as in animals. It is mostly seen in isolates of human infections, particularly in developing countries (Uzodi et al., 2017).

Serious staphylococcal infections in hospital and community settings are mostly caused by MRSA strains that cause a range of diseases, such as bacteraemia and skin and wound infections (Hassoun et al., 2017). This bacterium causes skin infections in pets, pigs, and mastitis in cattle. Human pathogenic MRSA strains have been identified in several animal species. People can contract specific strains of the virus by close contact with animals that carry it (Kinross et al., 2017).

One health strategy to fight AMR:

One Health strategy is completely included in international initiatives to deal with the AMR issue. The conflicting interests of numerous organizations and economic sectors concerned with animals, humans and the environment are just one among the challenges to be overcome. To effectively monitor antimicrobial resistance (AMR) and reduce infections, these actors must reach a consensus on important action priorities and policies governing the use of antibiotics. The followings are key tactics for tackling AMR:

1. Launch a global public awareness effort to inform people about the dangers of using antibiotics excessively and inappropriately. Reducing the number of antibiotic prescriptions can be achieved by implementing successful public campaigns.
2. To stop the escalation of illnesses, bolster and improve hygiene practices. Enhancing living conditions and healthcare systems will greatly lower the requirement for antibiotics, lowering the likelihood that a new resistant strain will emerge.
3. Minimize the needless application of antibiotics in farming and the environmental contamination they cause. The two industries that use the most antimicrobials worldwide are aquaculture and agriculture. Antimicrobials must not be utilized as growth boosters or as a preventative measure; they are harmful. Moreover, it is proved that a sizable portion of antimicrobials - between 75 and 90 percent - are excreted by animals without being digested and released into the environment.
4. Boost medication resistance monitoring worldwide. To comprehend the new processes of resistance acquisition, to identify existing cases with certainty, and to anticipate future risks, the scientific and medical community needs a thorough understanding of both historical and current data on AMR. To do this, a deeper comprehension of three areas is necessary: the molecular underpinnings of antimicrobial resistance (AMR), the usage of antibiotics by humans and animals, and the present rates of antibiotic resistance.
5. Encourage fresh, quick clinical diagnoses. Inappropriate antibiotic prescriptions are because of misdiagnoses made in both public and private hospitals. Antimicrobial therapy can be used in individuals who require it by clinicians with enhanced quick and precise diagnostic tests.
6. Encourage the creation and application of vaccinations and substitutes. The number of humans with infections requiring antimicrobial therapy will decline with the introduction of vaccinations targeted for bacteria that are antimicrobial resistant and can result in serious infections. New vaccines and antimicrobial substitutes, including phage treatment, probiotics, antibodies, and lysins, are currently in need of further funding.
7. Acknowledge and expand the pool of individuals handling infectious diseases. Experts in infectious disease, infection control, nursing, microbiology, pharmacy, veterinary medicine, and epidemiology are needed to address antimicrobial resistance (AMR). Countries must spend money on this human resource training in order to do this.
8. Form a worldwide alliance to take effective action against AMR. For the fight against AMR to make meaningful progress, global action is required. To bring about change, it is crucial to place AMR on the global political agenda and tackle it using a One Health approach (O'Neill et al., 2016).

Conclusion:

In order to combat AMR (people animal, plant, and environmental health), it is imperative to support a "One Health" policy. This means accelerating global development, investing in sustainable response, collaborating for more efficient action, strengthening global governance and accountability, and innovating to

safeguard the future. Most types of antibiotics are safe for use by humans and animals. AMR can be reduced by only treating infections with antimicrobials, rarely utilizing them for prophylaxis, and never using them as growth promoters. Success requires careful and effective control over the types and dosages of antimicrobials used in medical practice, as well as monitoring and controlling the development of environmental bacterial resistance.

Conflict of interest:

The authors declare that no conflict of interest exists.

Author's contribution:

Gurvinder: prepared the manuscript, and UJ reviewed the same Parul: draft preparation, BS: Material Resource, Aaryman: partial drafting, and Ravi: Revisions and critics.

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Isolation and Characterization of *Arcobacter* from Foods of Animal Origin and Environmental Samples

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Abstract

The present study was undertaken to optimize PCR assay for the detection of *Arcobacter* spp. And *Arcobacter butzleri* from foods of animal origin and environmental samples and compare its efficacy with conventional culture methods. Primers derived from 16S rRNA and 23S rRNA genes were used which gave specific amplification products of 181 bp and 686 bp for *Arcobacter* spp. and *Arcobacter butzleri,* respectively. Out of 240 samples screened (30 each of chicken, mutton, beef, raw milk, faecal samples of cattle, pig, and poultry, and water) for *Arcobacter* spp., PCR gave 31 isolates (chicken meat-6, mutton-4, beef-3, raw milk-2, faecal samples of cattle-4, faecal samples of pig-6 and faecal samples of poultry-5, water-1) as positive and culture method gave 21 isolates (chicken meat-4, mutton-3, beef-2, raw milk-1, faecal samples of cattle-3, faecal samples of pig-4 and faecal samples of poultry-4, water-nil) as positive. Out of 31 positive isolates by PCR, 15 (chicken meat-3, mutton-2, beef-2, raw milk-1, faecal samples of cattle-2, faecal samples of pig-2 and faecal samples of poultry-2, water-1) were positive for *Arcobacter butzleri*. *Arcobacter* spp.is an important emerging food and water-borne pathogens having public health concerns. Traditional methods for detecting and identifying *Arcobacter* spp. by culture methods are intensive and time-consuming, requiring 5-7 days to get a confirmed positive result. Hence there is a need to develop reliable and rapid methods for the detection of *Arcobacter* spp. from foods of animal origin.

Keywords: *Arcobacter*, Environmental samples, Isolation, Characterization, PCR assay.

Introduction:

Bacterial human pathogens continue to be a serious health issue and the prevalence of foodborne illnesses is still substantially high in the world. Although, *Arcobacter* spp. are not a member of the normal flora of the human intestine, humans can easily be infected due to the presence of the organisms in foods of animal origin and water. *Arcobacter*spp. are recognized as a potential food and waterborne pathogen (Gugliandolo etal., 2008;Miller etal., 2009).

Arcobacter genus belongs to the family *Campylobactereaceae*, which comprises two other genera *Campylobacter* and *Sulfurospirillum*. Consumption of *Arcobacter* contaminated food or water has been considered a mode of transmission. Water and food associated-outbreak reports strengthen their role in *Arcobacter* transmission routes (Lappi et al., 2013). In addition, the presence of *A. butzleri* and *A. cryaerophilus* in pet cats and dogs (Fera et al., 2009) suggests that contact with these animals can also be a potential route of human infection.

Currently, the pathogenic potential of *Arcobacter* spp. remain relatively unexplored with few studies concerned with clarifying the pathogenic mechanisms related to this genus. In-vitro human and animal cell culture assays have been used to show that several *Arcobacter* spp. can adhere to and invade eukaryotic cells and can produce toxins that damage host cells. As of today, several major questions regarding the *Arcobacter* genus remain unanswered, namely concerning genus diversity, its actual distribution that seems to be underestimated, and the virulence factors that are yet to be fully understood. It is opined that more species belonging to this genus are still to be recognized in the future. Also, the actual distribution of *Arcobacter* spp. may be underestimated, either because of a possible previous misidentification or due to the scarcity of works concerning the assessment of new species prevalence.

Materials and Methods:

Collection of samples: The specimens selected for this study were meat, milk, water, and faecal sample. Thirty chicken samples of each 50g were aseptically collected from local markets Directorate of Poultry Research (DPR) and All India coordinated Research project (AICRP) on poultry for eggs, Hyderabad. Thirty meat samples of 50g were aseptically collected from local market and modern slaughterhouse, Chengicherla. Thirty

beef samples of each 50g were aseptically collected from local markets. Thirty milk samples were collected aseptically in sterilized test tubes from local dairy farms, the College of Veterinary Science, Rajendranagar, Hyderabad, and local markets. Thirty Water samples of each 100 ml were collected from different sources like livestock farms of the College of Veterinary Science, Rajendranagar, Hyderabad, and local farms. Thirty each of cattle, pig, and poultry faecal samples of each 50g were aseptically collected from ILFC farm, College of Veterinary Science, Rajendranagar, Hyderabad, and Directorate of poultry research (DPR) and All India coordinated Research project (AICRP) on poultry for eggs, Hyderabad respectively.

Isolation and identification of *Arcobacter* spp.: *Arcobacter* enrichment broth with selective supplements (Cefoperazone and Amphotericin B) was used for the enrichment of samples. *Arcobacter* selective agar with selective supplements (Cefoperazone and Amphotericin B) and *Arcobacter* Blood agar plates were used for the isolation of *Arcobacter* spp.

Isolation: About ten (10) grams of each chicken, mutton, and beef sample were aseptically collected and suspended in 90ml of Phosphate buffer saline (PBS). The materials were homogenized thoroughly in a stomacher for 3 to 5 mins and incubated in *Arcobacter* enrichment broth at 30^0C under microaerophilic conditions for 48 hrs. One ml of milk and one ml of water were mixed with 9 ml of PBS and one gm of cattle, pig, and poultry faecal samples each were mixed with 9 ml of PBS, and samples were centrifuged at around 12000 RPM for 15 min and sediment was used for enrichment in *Arcobacter* enrichment broth. The inoculum from the broth after 48 hrs was streaked onto the *Arcobacter* selective agar plates and incubated at 30^0C for 48 hrs.

Identification: After homogenization of the faecal samples in 10ml PBS, 1ml of faecal suspension was inoculated into 9 ml *Arcobacter* Enrichment broth (1:9) with selective supplements and 5% defibrinated sheep blood for enrichment. Ten grams of meat samples were aseptically minced and suspended in 90ml of PBS, homogenized with a stomacher for 1 min at 200 rpm; 1 ml of suspension was inoculated into enrichment broth. Further incubation is done at 28^0C for 48 hrs under microaerophilic conditions for the enrichment of *Arcobacter*. These then were streaked onto *Arcobacter* selective agar plates with antibiotic supplements, and incubated microaerobically for 48-72 hrs. The suspected *Arcobacter* colonies were tested by gram staining, cellular morphology, catalase, oxidase test, and for motility using wet mount method. Up to 4-5 colonies were picked from each agar plate and sub-cultured on *Arcobacter* blood agar plates (*Arcobacter* broth fortified with 5% defibrinated sheep blood without selective antibiotic supplements and incubated aerobically at 28^0C for 48 hrs. A single colony was selected from blood agar plates for microscopic examination and biochemical tests.

Biochemical Characterization: For confirmation of *Arcobacter* spp. the biochemical tests conducted were oxidase test, catalase test, urease test, hippurate hydrolysis, and nitrate reduction test.

Polymerase Chain reaction:

Genomic DNA Extraction: Genomic DNA extraction from *A. butzleri* involved inoculation, centrifugation, lysis, proteinase treatment, phenol-chloroform extraction, precipitation, ethanol washing, and storage.

Preparation of template DNA from bacterial isolates: Preparation of template DNA from *Arcobacter butzleri* strains was carried out as per Lee (2013) with minor modifications. Loopful (3-5 suspected colonies) of 48 hrs. growth culture of the testorganism was suspended in 150 µl of nuclease-free water in a 0.5 ml microcentrifuge tube. After mixing properly the tubes were heated in a 100°C water bath for 15 min and immediately placed on ice. After 20 min the bacterial lysate was centrifuged at 13,000 RPM for 5 min and the supernatant was used as a DNA template for PCR assay for the detection of 16S rRNA and 23S rRNA genes.

Quantification and quality assessment of DNA: The quality and purity of DNA were checked by submarine agarose gel electrophoresis using 0.8% agarose in 0.5X TBE (pH 8.0) buffer. Ethidium bromide (1%) was added @ 5µl/100ml. The wells were charged with 5µl of DNA preparations mixed with 1µl of 6X gel loading buffer dye. Electrophoresis was carried out at 5V/cm for 20 min at room temperature and then the DNA was visualized under UV transilluminator. The quantity of DNA was calculated by spectrophotometric method. OD at 260 and 280 nm was taken in UV spectrophotometer with distilled water as reference. The purity of DNA was judged on the basis of the optical density ratio at 260:280 nm. The samples with acceptable purity (*i.e.* ratio 1.7-2.0) were used for PCR.

Oligonucleotide primers: The primers used from the16S rRNA gene and 23S rRNA gene for the detection of *Arcobacter* spp. and *Arcobacter butzleri* respectively were custom synthesized by Integrated DNA Technologies (IDT) are given in Table 1.

Optimization of the PCR protocol: PCR amplification of the 16S rRNA gene and 23SrRNA gene was set up to 25µl reactions. The PCR protocol was initially optimized by changing the concentration of the components of the reaction mixture in the PCR assay and by varying the annealing temperature and cycling conditions.

Reaction mixture: The components of the reaction mixture were finally optimized as 10X Taq polymerase buffer-2.5 µl, dNTP mix (each 1µl) - 4.0µl, Primer-F-1.0µl, Primer-R-1.0µl, and Purified DNA/Bacterial lysate-5.0 µl. The master mix was made up to 25µl using molecular grade water. Routinely, master mix was set up and 20µl each was distributed to the PCR tubes, to which 5µl of the template was added. In this study, the template preparation was done throughout the study by heat lysis application.

PCR assay was performed in an Eppendorf gradient Thermal cycler with a heated lid. The cycling conditions used are given in Table II. PCR products were stored at -20^{0}C until further use.Five µl of the bacterial lysate or 20 ng of purified DNA, 2.5µl of 10x assay buffer for Taq polymerase containing 1.5 mM $MgCl_2$, 1 µl of 25µM each dNTP mix, 1 µl each of forward and reverse primer (4pmol) and 1U/µl of Taq DNA polymerase made up to 25 µl using molecular grade water. Master mix was set up and 20 µl each was distributed to the PCR tubes, to which 5µl of the template was added.

Agarose gel electrophoresis: Agarose gel (1.5%) was prepared by boiling agarose in an appropriate volume of 1X TAE buffer. After cooling for about 3mins, ethidium bromide (Biogene, USA) was added to the agarose solution to a final concentration of 0.5 µg/ml. The molten agarose was then poured into the tray and the comb was fitted into the slots on the tray. The tray was kept undisturbed till the gel had solidified. The comb was then taken out carefully and the tray containing the gel was then placed in a submarine horizontal electrophoresis unit filled with 1X TAE buffer upto a level of 1mm above the gel surface.

About 5µl of each PCR product was mixed with 2µl of bromophenol blue (6X) loading dye and loaded into each well. Electrophoresis was performed at 5 V/cm and the mobility was monitored by the migration of the dye. After sufficient migration, the gels were observed under a UV transilluminator to visualize the bands. The PCR product size was determined by comparing it with a standard molecular weight marker and was photographed by the gel documentation system.

Results and Discussion:

A total of 240 samples comprising each 30-chicken meat, mutton, beef, pork, raw milk, water, and faecal samples of cattle, poultry, and pig were collected and analyzed for the presence of *Arcobacter* and *Arcobacter butzleri*.

Isolation of *Arcobacter* spp.: All the samples collected were inoculated into Arcobacter enrichment broth and incubated at 28^{0}C for 48 hrs. microaerobically. After the incubation

period was over a loopful of inoculum from *Arcobacter* enrichment broth tubes was streaked on *Arcobacter* selective agar plates with added supplements by following all the aseptic precautions and then plates were incubated at 28^{0}C for 48-72 hrs. microaerobically. The plates were observed for characteristics translucent to whitish colonies and were selected as suspected *Arcobacter* (Figure 1 and 2).

Figure 1: Culture characteristics of *Arcobacter*-like isolates on Arcobacter selective agar

Table 1: Details of Oligonucleotide primers used in the study

Primer	Target Gene	Length	Primer sequence (5'-3')	Amplification product (bp)	Reference
Arc-1	16S rRNA	22	AGAACGGGTTATAGCTTGCTAT	181bp	Harmon and Wesley (1996)
Arc-2		22	GATACAATACAGGCTAATCTCT		
Arc-5	23S rRNA	17	TTCGCTTGCGCTGACAT	686 bp	De Oliveira et al. (1997)
Arc-6		19	CTATCCAGCGTAGAAGATG		

Table 2: Cycling conditions used for two sets of primers

Sl. No.	Step	16S rRNA	No. of cycles	23S rRNA	No. of cycles
1.	Initial denaturation	94^{0}C/3min 30sec	1	95^{0}C/30sec	1
2.	Final denaturation	94^{0}C/1min	32	94^{0}C/1.15min	40
3.	Annealing	55.6^{0}C/1min		60.7^{0}C/45sec	
4.	Initial extension	72^{0}C/1min		72^{0}C/1min 30sec	
5.	Final extension	72^{0}C/5min	1	72^{0}C/5min 54sec	1
6.	Hold	4°C	-	4^{0}C	-

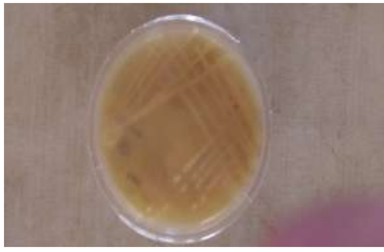

Figure 2: Culture characteristics of *Arcobacter* isolates on BHI agar

The isolates were subjected to Gram's staining and found Gram-negative (Figure 3), spirally curved rod or "S" shaped with rapid cork screw like motility. These suspected *Arcobacter* were streaked on Arcobacter blood agar plates with selective supplements for further biochemical confirmation.

Biochemical tests: The biochemical reactions of all the isolates were given in Table 3. The isolates were subjected to the biochemical tests like catalase test, oxidase test (Figure 4), hippurate hydrolysis, nitrate reduction tests and indoxyl acetate hydrolysis. All the isolates were positive for oxidase test producing deep purple colour and positive for catalase test.

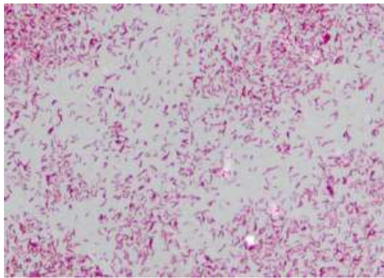

Figure 3: Gram's staining of *Arcobacter* isolates

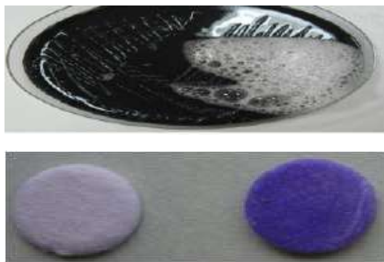

Figure 4: Positive results of Catalase test and Oxidase test with *Arcobacter* isolates

In recent years, a significant amount of progress has been made in understanding the ecology, prevalence and clinical implications of *Arcobacter* spp. infection from a food safety perspective (Shah et al., 2014). Evidence clearly indicates the association of *Arcobacter* spp. with animal foods and human illness, even though the transmission routes of *Arcobacter* spp. infection to human beings is not fully elucidated. The incidence of *Arcobacter* in chicken meat than in the present study (13.3%) by culture method *i.e.,* 10% was reported by Mohan et al. (2014), whereas Ramees et al. (2014) reported higher incidence (21.85%). The incidence of *Arcobacter* by PCR method in the present study (20%) was nearer to the findings (23%) of Kabeyaet al. (2004). The incidence of *A.butzleri* in the present study (50%) by PCR was less than the incidence (73%) reported by Rivas etal.(2004) and higher than the incidence(19.64%) reported by Bagalkote et al. (2013).

Optimizing of PCR assay: The PCR assay for detecting *Arcobacter* spp. and *A. butzleri* was optimized using primers targeting the 16S rRNA and 23S rRNA genes. Optimal conditions included specific annealing temperatures, primer concentrations, and cycling parameters resulting in the amplification of specific fragments.

Screening of natural samples: The results of the samples by culture and PCR method are, In the case of chicken samples out of 30 samples collected, 4(13.3%) samples were positive by culture method and 6(20%) samples were positive by PCR assay, among the 6 samples, 3(10%) were positive for *A. butzleri* by PCR assay. In mutton samples collected, 3(10%) samples were positive by culture method and 4(13.3%) samples were positive by PCR assay, among the 4 samples 2(6.6%) were positive for *Arcobacter butzleri* by PCR assay. In the case of beef samples out of 30 samples screened, 2(6.6%) samples were positive by culture method and 3 (10%) samples were positive by PCR assay, among the 3 samples 2 (6.6%) were positive for *A. butzleri* by PCR assay. Out of 30 Raw milk samples screened for the presence of *Arcobacter* spp., 1(3.3%) sample was positive by culture method and 2(6.6%) samples were positive by PCR assay, among the 2 samples, 1(3.3%) sample was positive for *A. butzleri* by PCR assay. Out of 30 cattle faecal samples screened for the presence of *Arcobacter* spp., 3(10%) samples were positive by culture method and 4(13.3%) samples were positive by PCR assay, among the 4 samples, 2(6.6%)samples were positive for *A. butzleri* by PCR assay. Out of 30 poultry faeces samples screened for the presence of *Arcobacter* spp. in this study, 4(13.3%) samples were positive by culture method and 5(16.6%) samples were positive by PCR assay, among the 5 samples 2(6.6%) were positive for *A.butzleri* by PCR assay. Out of 30 pig faecal samples screened for the presence of *Arcobacter* spp. 4(13.3%) samples were positive by culture method 6(20%) samples were positive by PCR assay among the 6 samples, 2(6.6%) samples were positive for *A. butzleri* by PCR assay.

The incidence of *Arcobacter* in the present study by PCR in mutton samples (13.34%) were almost similar to the incidence (15%) reported Rivas et al. (2004). Low incidence of *Arcobacter* in beef by culture method than the present study (16%) was reported by Mohan et al. (2014). The incidence of *A. butzleri* in the present study (66.6%) by PCR was less than the incidence (100%) reported by Mohan et al. (2014).

Low incidence of *Arcobacter* in raw milk than the present study (3.3%) by culture method *i.e.*, 1% and 1.67% was reported by Ramees et al. (2014) and Mohan et al. (2014) respectively. The incidence of *Arcobacter* by PCR method in the present study (6.6%) are similar to the findings (6%) of Ertas et al. (2010) and lower than the incidence (1.67%) was reported by Mohan et al. (2014). The incidence of *A. butzleri* in the present study (50%) by PCR was nearer to the findings (46%) reported by Scullion et al. (2006) and less than the incidence (0.53%) reported by Pinata et al. (2007). Mohan et al. (2014) reported (10%) almost nearer to results obtained in this study. The incidence of *Arcobacter* by PCR method in the present study (50%) was higher than the incidence (37.3%) reported by Fernandez et al. (2015). The incidence of *A. butzleri* in the present study (50%) was less than the incidence (65%) reported by Van dreissche and Houf, (2003) and higher than the incidence (4%) reported by Aydin et al. (2007).

Low incidence of *Arcobacter* in poultry faecal samples than in the present study (13.3%) by culture method *i.e.* 8% was reported by Mohan et al. (2014) whereas; Ramees et al. (2014) reported a higher incidence (21.85%). The incidence of *Arcobacter* by PCR method in the present study (16.6%) was similar to the findings (17.3%) of Patyal et al. (2011) and higher than the incidence (10%) reported by Mohan et al. (2014). The incidence of *A. butzleri* in the present study (40%) by PCR was higher than the incidence (14.8%) reported by Mohan et al. (2014).Slightly low incidence of *Arcobacter* in pig faecal samples,thanin the present study (13.3%) by culture method *i.e.,* 12% was reported by Mohan et al. (2014). The incidence of *Arcobacter* by PCR method in the present study (20%) was lower than the findings (59.2%) of by Fernanadez et al. (2015). The incidence of *A. butzleri* in the present study (33.3%) by PCR was less than the incidence (80.5%) reported by Van Driessche and Houf, (2003). The incidence of *A. butzleri* in the present study (100%) was higher than incidence (68%) reported by Jacob et al. (1998) and zero incidence was reported by Aydin et al. (2007).

Table 3: Results of biochemical tests performed for confirmation of *Arcobacter* isolates.

Sl. No.	Name of the biochemical tests	Number of isolates positive for the bio-chemical and other tests							
		Chicken	Mutton	Beef	Raw milk	Cattle faeces	Poultry faeces	Pig faeces	Water
1	Gram's staining	4	3	2	1	3	4	4	0
2	Motility	4	3	2	1	3	4	4	0
3	Catalase test	4	3	2	1	3	4	4	0
4	Oxidase test	4	3	2	1	3	4	4	0
5	Hippurate Hydrolysis	0	0	0	0	0	0	0	0
6	Nitrate reduction test	4	3	2	1	3	4	4	0
7	Indoxylacetate hydrolysis	4	3	2	1	3	4	4	0
8	Urease test	0	0	0	0	0	0	0	0
9	H_2S productn on TSI	0	0	0	0	0	0	0	0

Conclusions:

Traditional methods for detecting and identifying *Arcobacter* spp. by culture methods are intensive and time-consuming, requiring 5-7 days for getting a confirmed positive result. Hence there is a need to develop reliable and rapid methods for detection of *Arcobacter* spp. from foods of animal origin.

Further scope of research:

There is scope to identify the role and significance of each *Arcobacter* spp. especially where co-infections occur with other bacteria in livestock.

Conflict of interest:

The authors declare that they have no conflict of interest.

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Characterization of Extended-spectrum and pAmpC Beta-lactamases producing *Escherichia coli* isolated from Chicken Meat in West Bengal

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Abstract

Escherichia coli is one of the pathogenic bacteria causing food-borne infections like chicken meat, which can lead to serious public health hazards. Antimicrobial resistance in *E. coli* is a serious threat to the human population nowadays. The present study aimed to detect extended-spectrum beta-lactamases (ESBLs) and AmpC beta-lactamase (ACBL)-producing *Escherichia coli* from chicken meat, from different districts of West Bengal. A total of 107 raw chicken meat samples were collected just after slaughter from different districts' local markets followed by isolation and identification of 74(69.16%) *E. coli* isolates by standard conventional and molecular methods. Among the positive isolates, 23(31.08%) were positive to ESBL production with the presence of the $bla_{\text{CTX-M}}$ gene, whereas 65(87.84%) strains were found to possess the bla_{AmpC} gene. Antibiogram study of ESBL-positive *E. coli* strains revealed the sensitivity of these strains to imipenem (65.65%), gentamicin (86.96%), ampicillin/sulbactam (78.26%), and amikacin (82.61%) whereas all other antimicrobials were resistant against these pathogens.

Keywords: Antibiogram, ACBL, CTX-M, Chicken meat, *E. coli*, ESBL

Introduction:

Meat production in India is growing day by day. Now, the annual meat production of India is 5.3 million metric tons which is the 5th largest in the World (DAHD, 2017). India has the world's largest livestock population which plays an important role in rural economy and livelihood. It produces 21% of global chicken meat production annually. The poultry industry is rapidly growing in India as well as in the state of West Bengal. West Bengal is the 2nd largest contributor with 640 thousand metric tons of meat production of which chicken meat production is 328 thousand metric tons (DAHD, 2017).But chicken meat can easily get spoilt with bacterial spoilage due to faulty handling, improper storage, and poor management of the birds (Dierikx et al., 2010).

Antimicrobial resistance (AMR) is increasing day by day. With the increased consumption of antibiotics, in the last few decades leading to a rise in their resistance among microbial populations. The incidence of extended-spectrum beta-lactamases (ESBLs)-producing *Escherichia coli* in food is quite significantly increasing nowadays all around the World. ESBL production in bacteria is governed by the presence of $bla_{\text{CTX-M}}$, bla_{SHV}, and bla_{TEM} genes which are easily transferred from one bacterium to another spreading the antimicrobial resistance. Among these resistance genes, the $bla_{\text{CTX-M}}$ gene is the most common gene associated with ESBL positivity in *E. coli* (Dierikx et al., 2010). This drug-resistant pathogen can create a major problem during their treatment forcing the clinicians to use newer and newer antibiotics (Tenover et al., 1999; Olesen et al., 2004).These antimicrobial resistance genes of *E. coli* are easily transferrable to other pathogens conferring them resistance. The AmpC beta-lactamase (ACBL) is the first bacterial enzyme reported to destroy penicillin in Gram-negative bacteria like *Escherichia coli*. ACBL encoding gene bla_{AmpC} is found in transmissible plasmids and also in bacterial chromosomes (Reich et al., 2013). ACBL-producing *E. coli* strains are resistant to broad-spectrum cephalosporins but their resistance patterns are less expressed *in-vitro* than that of the ESBLs (Jacoby, 2009).

Common people consider chicken meat as a very popular source of animal protein worldwide. ESBL-producing *E. coli* is frequently reported from chicken samples worldwide and may be pathogenic to humans causing urinary tract infections, septicemia, meningitis, etc. (Grami et al., 2013; Nandanwar et al., 2014). Most of the countries are using a large quantity of different antimicrobials to raise poultry which are also used in human treatments. Indiscriminate use of such essential antimicrobials in animal production is likely to accelerate the resistance development of pathogens, as well as commensal organisms like *E. coli*. This would result in

treatment failures and economic loss and could act as a source of the gene pool for transmission to humans (Castanon, 2007). In addition, there are human health concerns about the presence of antimicrobial residues in meat, eggs, and other animal products (Sahoo et al., 2010; Darwish et al., 2013).

Identification of potential MDR pathogenic bacteria is essential towards the development of better managemental procedures. With this background, the present research has aimed at the detection and characterization of ESBL and ACBL-producing *E. coli* from raw chicken meat collected from different local markets of West Bengal and followed by an *in-vitro* antibiogram to assess their resistance patterns.

Materials and Methods:

Sample collection: A total of 107chicken meat samples were collected at the time of slaughter from local markets of Paschim Medinipur, Nadia, and Hooghly district of West Bengal during the period of August to December 2022. Ten grams of fresh meat samples were aseptically collected in individual vials and transported (under ice cover) to the laboratory. Samples were enriched on the date of receiving in the laboratory.

Bacteriological isolation and characterization: A 10% homogenized suspension of each meat sample was prepared in sterile normal saline and streaked onto Mac Conkey's agar (Hi-Media, India) and then onto EMB agar (Hi-Media, India) plates followed by incubation overnight at 37°C. The chocolate colour colonies with a 'metallic sheen' were picked up for further morphological (by Gram's staining) and biochemical characterization (Carter and Wise, 2004; Quinn et al., 2011). One tentative *E. coli* isolate from each sample was taken in this study.

Confirmation of *Escherichia coli* by PCR: All the tentative *E. coli* isolates were confirmed by detection of the 16S*rRNA* gene specific for this genus, following the protocol of Wang et al., 1996 (Table 1).

Phenotypic detection of ESBL in *E. coli* strains: Phenotypic detection of ESBL activity of the *E. coli* isolates was done by double disc diffusion assay (Bauer et al., 1966) using both cefotaxime (30µg) and ceftazidime disks (30µg) and their clavulanate (10µg) discs as per CLSI method of Patel et al. (2015). An increase of zone diameter (>5mm) in each clavulanate disk than the single drug disk is treated as phenotypical confirmation of the ESBL activity.

Molecular detection of ESBL positivity: All the *E. coli* isolates were screened for ESBL positivity by the detection of the $bla_{\text{CTX-M}}$ gene by PCR assay as per the protocol of Weill et al. (2004) (Table 1). In this method, 5µl bacterial DNA templates, 50pmol of each primer, 200mM deoxynuc1eoside triphosphate, 1U Taq DNA polymerase (Promega, USA), 2mM $MgC1_2$, and 10% dimethyl sulfoxide (DMSO) was added in a 25µl reaction mixture and subjected to amplification with following PCR conditions - 10mins of initial denaturation at 94°C followed by 30s of denaturation at 94°C, 30s of annealing at 53°C and 1min of extension at 72°C for 35 cycles and 10mins of final extension at 72°C. The amplified product was visualized by gel documentation system (UVP, UK) after electrophoresis in 1.5% (w/v) agarose (SRL, India) gel containing ethidium bromide (0.5µg/ml) (SRL, India). In this study, an *Escherichia coli* serotype O2, maintained in the departmental laboratory was used as the positive control.

Phenotypical detection of ACBL Production in *Escherichia coli* isolates: *In-vitro* ACBL activity of all *Escherichia coli* isolates was examined following the protocol of Tan et al.(2009) by cefoxitin–cloxacillin double-disc synergy (CC-DDS) test.

PCR detection of *AmpC* gene in *E. coli* strains: All the *E. coli* strains were examined for the presence of the AmpC gene by PCR following the protocol of Feria et al., 2002 (Table 1). In this method, the total reaction volume was 25µl containing 5µl of bacterial DNA template, 100 pmol of each primer, 200 mM of each dNTP, 2mM $MgCl_2$, and 10% DMSO. The PCR mixture was subjected to an initial denaturation step of 5mins at 94°C; followed by 30cycles of amplification consisting of 30s of denaturation at 94°C, 30s of annealing at 57°C, 1min of elongation at 72°C and 10mins of final extension at 72°C. The PCR product was electrophoresed in 1.5% (w/v) agarose (SRL, India) gel containing ethidium bromide (0.5µg/ml) (SRL, India) and the gel was visualized in a gel documentation system (UVP, UK).

Antibiogram of ESBL-positive *E. Coli*s strains: *In-vitro* antibiotic sensitivity of the ESBL gene-positive *E. coli* isolates was examined using 10 commonly used antimicrobials *viz.* ampicillin, amikacin, ampicillin/ clavulanic acid, ampicillin/ sulbactam, cefotaxime, ceftriaxone, ceftazidime, gentamicin, imipenem, and norfloxacin by disc diffusion method (Bauer et al., 1966). Standard antibiotic discs (Hi-Media, India) were used as the source of antibiotics. The inhibition zone diameters were interpreted following the standard chart (Patel et al., 2015).

Table 1: Details of Target Genes and their Primers used in this Study			
Gene	Primer sequence	Size	Reference
E. coli 16S *rRNA*	ECO-1 F 5'GACCTCGGTTTAG TTCACAGA3'ECO-2 R 5'CACACGCTGACG CTGACCA3'	585 bp	Wang et al., 1996
bla$_{CTX_M}$ consensus (ESBL)	CTX-M F 5' CCATGTGCAGCACC AGTAA 3' CTX-M R 5' CGCAATATCCTTGG TGGTG 3'	540 bp	Weill et al., 2004
bla$_{AmpC}$ (ACBL)	AmpC F 5'CCCCGCTTATAGA GCAACAA3'AmpC R 5'TCAATGGTCGACT TCACACC3'	634 bp	Feria et al., 2002

Results and Discussion:

Approximately 74 (69.16%) chicken meat samples were tested to be positive for *Escherichia coli* in this study. All the isolates of *E. coli* were characterized by pinkish colonies on Mac Conkey's agar, characteristic 'metallic sheen' on EMB agar plates, Gram's negative rods, and positive reaction to the indole test. All the isolates were detected to possess the 16S *rRNA* gene (Figure 1) sterile EMB agar (Hi-Media, India) plates and thus confirmed to be *E. coli* (Carter and Wise, 2004; Quinn et al., 2011; Samanta, 2013). This study identified a very high prevalence (78.86%) of *Escherichia coli* in poultry meat, which matches with earlier works of Reich et al. (2013), Maciuca et al. (2015), and Klimiene et al. (2018) who reported 45%, 54% and 92% *E. coli* prevalence in chicken meat from different countries showing the significant presence of the pathogen in the food chain (Dierikx et al., 2010). Samples from the Hooghly district were mostly infected (79.17%) followed by Paschim Medinipur and Nadia (Table 2).

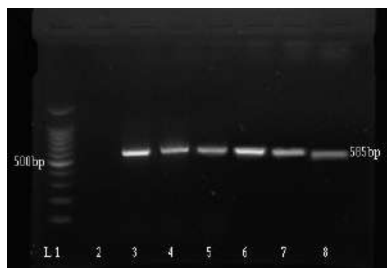

Figure 1: Detection of the 16S *rRNA* gene (585bp) in *E. coli* isolates by PCR (Lane 1: 100bp ladder, L2: uninoculated negative control, L3: positive control, L4-8: test samples)

A total of 18(24.32%) *E. coli* isolates were detected to be phenotypical ESBL producers; whereas 23(31.08%) isolates were found to possess the *bla*$_{CTX-M}$ gene (Figure 2) by PCR. Again 57(77.03%) strains were positive to ACBL production and a total of 65(87.84%) *E. coli* isolates were confirmed to possess the *bla*$_{AmpC}$ gene genotypically (Figure 3, Table 2). Seventeen *E. coli* strains had both the genes (Table 2). Such higher ESBL positivity in poultry *E. coli* isolates has also been reported from different countries by Maamar et al. (2016), Tekiner and Ozpinar (2016), Casella et al. (2017) and Klimiene et al. (2018). The gene *bla*$_{CTX-M}$ is the most common and dominant gene among all ESBL genes (Feria et al., 2002; Samanta, 2013). The present study also confirms the earlier reports and identifies it as a potential threat to even human health (Dierikx et al., 2013). The *AmpC* beta-lactamase enzyme is also highly prevalent (87%) among the *E. coli* strains, and the prevalence rate was higher than the observations of Casella et al., 2017 (3.9%) and Van et al., 2008 (23.7%).

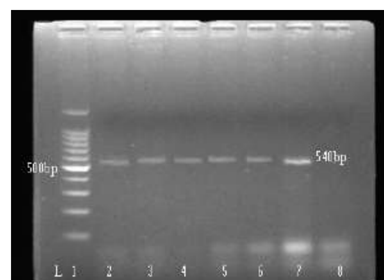

Figure 2: Molecular detection of the *bla*$_{CTX-M}$ gene (540bp) in *E. coli* isolates by PCR (Lane1: 100bp ladder, L2-6: test samples, L7: positive control, L8: uninoculated negative control)

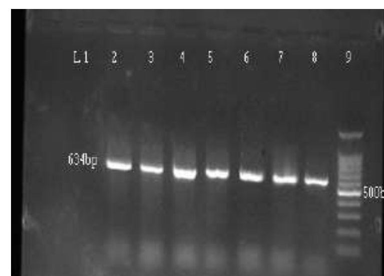

Figure 3: PCR Detection of the *bla*$_{AmpC}$ gene (634bp) in *E. coli* isolates by PCR (Lane 1: uninoculated negative control, L2: positive control, L3-8: test samples, L9: 100bp ladder).

In-vitro antibiogram of the ESBL-positive *E. coli* isolates revealed high-level resistance (74-100%) to ampicillin,

ceftriaxone, cefotaxime, ceftazidime, ampicillin-clavulanic acid, and norfloxacin (Table 3). In contrast, isolates were sensitive to drugs like amikacin, gentamicin, imipenem, and ampicillin-sulbactam. Such high-level drug resistance by the ESBL-positive *E. coli* isolates was also reported by Van et al. (2008), Reich et al. (2013), Beninati et al. (2015) and Maamar et al. (2016). Again, Tekiner and Ozpınar (2016) reported that *E. coli* from raw chicken meat were resistant to cefotaxime (62.1%), ceftazidime (55.2%), cefoperazone (51.7%) and cloxacillin (20.6%). Van et al. (2008) also reported multidrug resistance among poultry meat *E. coli,* which were resistant to tetracycline (77.8%), ampicillin (50.5%), gentamicin (24.2%) and norfloxacin (17.2%) although few other studies indicated that ESBL positive *E. coli* strains are sensitive to few beta-lactams and aminoglycosides like amikacin, imipenem, ampicillin-sulbactam, and gentamicin (Tekiner and Ozpınar, 2016; Castillo et al., 2018). The rapid increase in the development and spread of antimicrobial resistance (AMR) is a matter of serious concern (Van et al., 2008; Ryu et al., 2012).

Table 2: Detection and distributions of *beta*-lactamases producing genes among *E. coli* isolates from chicken meat in West Bengal

Name of the Districts	No. of meat samples screened	No. of *E. coli* strains Isolated (%)	ESBL positivity in *E. coli* strains (%)	ACBL Positivity in *E. coli* strains (%)	Gene distribution in positive *E. coli* strains		
					bla$_{CTX-M}$ only	*bla*$_{AmpC}$ only	*bla*$_{CTX-M}$ + *bla*$_{AmpC}$
Paschim Medinipur	37	26 (70.27)	6 (23.07)	21 (80.77)	2	18	5
Nadia	46	29 (63.04)	7 (24.14)	23 (79.31)	3	19	6
Hooghly	24	19 (79.17)	5 (26.31)	13 (68.42)	1	11	6
Total	107	74 (69.16)	18 (24.32)	57 (77.03)	6	48	17

Table 3: Resistance pattern of 23 ESBL-positive *E. coli* isolates obtained from chicken meat in West Bengal

Sl. No.	Antimicrobials (Conc. in µg)	Isolates sensitive		Isolates intermediately sensitive		Isolates resistant	
		No.	%	No.	%	No.	%
1.	Amikacin (AK - 30)	19	82.61	4	17.39	0	0
2.	Ampicillin / Clavulanic acid (AMC - 30)	0	0	1	4.35	22	95.65
3.	Ceftriaxone (CTR 30)	0	0	0	0	23	100
4.	Ampicillin/Sulbactam (*A/S* - 10/10 mcg)	18	78.26	5	21.74	0	0
5.	Ampicillin (AM - 10)	0	0	0	0	23	100
6.	Ceftazidime (CAZ - 30)	0	0	1	4.35	22	95.65
7.	Imipenem (IPM - 10)	22	95.65	1	4.35	0	0
8.	Gentamicin (GEN - 10)	20	86.96	3	13.04	0	0
9.	Norfloxacin (NX - 10)	0	0	6	26.09	17	73.91
10.	Cefotaxime (CTX - 30)	0	0	1	4.35	22	95.65

Conclusion:

It can be concluded that approximately 69% of the chicken meat samples screened in this study, were found to be positive for *E. coli* strains. About 31% and 87% of these bacteria were positive for ESBL and ACBL production which are quite significant data. The ESBL-positive stains were resistant to most commonly used antimicrobials other than amikacin, imipenem, gentamicin, and ampicillin/sulbactam. These drug-resistant *Escherichia coli*s train scan become a serious concern for causing animal and human health.

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Conflict of Interest:

No competing interest exists among the authors.

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Unveiling the Cultivation Dynamics of Thermophilic Campylobacters in Different Culture Media Substrate

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Abstract

Campylobacter species, known for their potential zoonotic transmission, play a significant role in instigating enteritis across a diverse array of animals, including those in captive, wild, and domestic environments, alongside avian populations. We isolated *Campylobacter jejuni* and *Campylobacter coli* strains from faecal specimens sourced from wild animals. The sample collection encompassed 521 faecal samples procured from wildlife sanctuaries, zoos, and national parks situated in Uttar Pradesh, Uttarakhand, and Chhattisgarh. Employing a meticulously aseptic approach throughout the collection and isolation process, the growth patterns of the identified *Campylobacter* strains were meticulously scrutinized using five distinct artificial culture media, classified into two groups: blood-free and blood-containing media. The prevalence of Campylobacter spp. was observed to be 11.90% (62 out of 521 samples), with *Campylobacter jejuni* accounting for 7.10% and *Campylobacter coli* for 4.80% of the isolates. Subsequent enrichment and plating on Columbia Blood Agar (CBA) supplemented with selective additives demonstrated a notably higher prevalence (11.90%) of *Campylobacter* spp. compared to alternative media such as Modified Charcoal Cefoperazone Deoxycholate Agar [mCCDA] (10.56%) and Sheep Blood Agar [BA] (8.25%). The culture of *Campylobacter* strains exhibited the lowest isolation rates on Chocolate Agar [CA] (5.76%) and Hi-chrome *Campylobacter* Agar [HCCA] (4.22%). The findings from multiplex PCR assays confirmed both the precise identification of Campylobacter species and the efficacy of each culture method employed in this study.

Keywords: *Campylobacter* spp., Culture media, Multiplex PCR, Prevalence.

Introduction:

Campylobacter species are fastidious organisms that have specific atmospheric and temperature requirements for growth, utilize menaquinones as their respiratory quinones, do not ferment or oxidize carbohydrates, and thrive in a microaerophilic (5% O2, 10% CO2, and 85% N2)environment (Penner,1988) Campylobacter strains causing human gastroenteritis are predominantly thermotolerant and capable of growing at temperatures as high as 42°C–43°C (Vandamme and De Ley, 1991).Campylobacter is a significant zoonotic foodborne bacterial pathogen that causes diarrheal diseases in both humans and animals (Garcia-Sanchez et al., 2018; WHO, 2020). *Campylobacter* is one of the most prevalent bacterial agents responsible for gastroenteritis, although the true incidence of *Campylobacter*-related gastroenteritis, particularly in low- and middle-income countries (LMIC), remains poorly understood, with estimates indicating around 3 cases per 1000 population (WHO, 2012). Transmission of the pathogen to humans can occur through various routes, including contaminated food, water, and direct contact with farm animals and pets (Elbrissiet al., 2017).

Materials and Methods:

We collected 521 faecal samples from wild animals, including mammals and birds between April 2021 and March 2022 from eight zoos, national parks, and sanctuaries located in Uttarakhand (n=3), Uttar Pradesh (n=2), and Chhattisgarh (n=3), India (Table 1).

Samples were immediately processed following the guidelines of ISO 10272-1:2017(E). The samples underwent pre-enrichment in Buffered peptone water (BPW) and then enrichment was performed using Bolton broth supplemented with 5% sterile lysed defibrinated sheep blood and FD231 supplement. The enriched samples were incubated microaerobically in a CO_2 incubator at 42°C for 48 hours. After the primary isolation of Campylobacter species, five different artificial media were assessed, and categorized into two groups: blood-free media and blood-containing media.

The blood-free media included Modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) and Hi-chrome *Campylobacter* agar (HCCA). The blood-containing media consisted of Columbia blood agar (CBA), Sheep blood agar (BA), and chocolate agar (CA).

Table1: Places of faecal samples collection and description of animals					
Sl. No.	**Places of sample collection**	**No. of faecal samples collected**	**Ruminants**	**Non-Ruminants**	**Birds**
1.	Deer Park and Wild Animal Rescue Center, NTD, Almora, Uttarakhand, India	24	6	18	0
2.	G. B. Pant High Altitude Zoo Nainital, Uttarakhand, India	32	11	18	3
3.	Jim Corbett National Park, Ramnagar, Nainital, Uttarakhand, India	138	138	0	0
4.	Kanpur Zoological Park, Nawabganj Kanpur, Uttar Pradesh, India	34	10	10	14
5.	Nawab Wajid Ali Shah Zoological Garden Lucknow, Uttar Pradesh, India	11	4	3	4
6.	State Nandanban Zoo & Safari, New Raipur, Chhattisgarh, India	76	22	48	6
7.	Peripherry of Achanakmar Sanctuary, Bilaspur, Chhattisgarh, India	99	51	33	15
8.	State Zoo, Bilaspur, Chhattisgarh, India	107	60	36	11
	Total	**521**	**302**	**166**	**53**

Following pre-enrichment and enrichment, the obtained isolates were inoculated onto mCCDA, HCCA, CBA, BA, and CA media and incubated in a CO_2 incubator maintained at 5% CO_2 and 42°C. Incubation was carried out for a period of 48-72 hours.

Suspected and well-isolated colonies were subcultured onto the same media for purification. Gram staining and standard biochemical tests, including oxidase test, catalase test, Hippurate hydrolysis test, *Campylobacter* nitrate reduction test, urease test, and H_2S production on TSI test methods, were performed for further identification of the presumed colonies (Atabay and Corry, 1997). Biochemically positive isolates were grown in Tryptone soya broth, aliquoted into cryo-vials with 20% sterile glycerol, and preserved at -80°C for future use.

Positive isolates, based on colony appearance and biochemical results, were further confirmed through multiplex PCR following DNA extraction (Shams et al., 2017). The prevalence data of *Campylobacter* spp. recovered from each culture medium were statistically compared using one-way analysis of variance followed by the least significant difference (DMRT) test. The statistical analyses were performed using SPSS version 26.

Results and Discussions:

The identification of suspected *Campylobacter* isolates was based on their colony characteristics, motility test, inability to grow in aerobic conditions, and Gram staining features. *Campylobacter* colonies exhibited small (1-2 mm), circular, flat to slightly raised, sticky, spreading, shiny grey-coloured colonies or water droplets on mCCDA, CBA, BA, and Chocolate agar plates. On HCA plates, *Campylobacter* species appeared as mauve to purple-coloured colonies. The organisms appeared as pink Gram-negative rods, spiral curved rods with comma-shaped (S) or gull-wing appearance cells. Similar colony characteristics were reported by Monika (2014) and Garhia(2017).

The recovery rate of *Campylobacter* spp. in this study was higher in CBA culture media compared to mCCDA, HCA, BA, and Chocolate agar culture methods studied (Table 2). The overall prevalence of *Campylobacter* spp. was 11.90% (62 out of 521), with *Campylobacter jejuni* accounting for 7.10% and *Campylobacter coli* for 4.80%, which aligns with the findings of Acke et al. (2008). After enrichment, plating on CBA with selective supplement resulted in a significantly higher ($P<0.05$) prevalence of 4.65% of *Campylobacter* spp., as also reported by Hutchinson and Bolton (1984). However, we observed a recovery rate of 10.56% on mCCDA, as reported by Corry and Atabay (1997), and 8.25% on BA (Byrne et al., 2001), showing no significant differences (Table 2), followed by 5.76% on CA, as also reported by Aspinall et al. (1996), and 4.22% on HCCA (Humphrey et al., 2007). Multiplex PCR results confirmed the speciation of *Campylobacter* isolates as well as the sensitivity of each culture method. Considering the majority of *Campylobacter* spp. was isolated using CBA media with a selective supplement, it can be concluded that this method is preferable for the isolation of *Campylobacter* sp. in this study.

Table 2: *Campylobacter* spp. prevalence for each culture and combined method (%)		
Sl. No.	**Culture Methods**	***Campylobacter* species prevalence (%)**
1.	mCCDA	55/521 (10.56)[d]
2.	Hi-chrome CA (HCCA)	22/521 (4.22)[a]
3.	CBA	**62/521 (11.90)[e]**
4.	BA	43/521 (8.25)[c]
5.	Chocolate Agar (CA)	30/521 (5.76)[b]
6.	Total	**62/521(11.90)**
7.	C.D.	0.075
8.	SE (m)	0.023
9.	SE (d)	0.033
10.	C.V.	0.627

* Figures having different superscripts differ significantly. (P=1.42e-12) P<0.05

It was also observed that the pre-enrichment and enrichment steps reduced transport stress and enhanced the recovery of *Campylobacter* spp. compared to direct plating or filtration onto selective media. Since CBA showed a higher recovery rate of *Campylobacter* spp. (P<0.05), it may be considered a more accurate blood-based method for assessing the actual prevalence of *Campylobacter* spp. in the sampled population. In the blood-free method, mCCDA may be relatively better for assessing the prevalence of *Campylobacter* spp. In both methods, CBA and mCCDA, hemin (Fe^{3+}) and charcoal, respectively, act as a source of energy and oxygen-quenching agent, which are necessary for growth in a microaerophilic environment (Hutchinson and Bolton, 1984).

Conclusions:

The recovery of *Campylobacter spp*. is very tedious and time-consuming task owing to the presence of multifaceted micro-flora in faecal samples as well as fastidious and microaerophilic nature of *Campylobacter* spp. It takes 3-5 day in confirmation of a faecal sample. For isolation of *Campylobacter species* from faecal samples of wild animals pre-enrichment in PBS and enrichment in Bolton broth as well as CBA selective media were found very suitable method for accurate prevalence assessment. In India, majority outbreaks of foodborne disease go unreported, unrecognized or un-investigated and may only be noticed after major health or economic damage has occurred. In such a condition controlling the outbreaks, detection and removal of implicated foods, identification of the factors that contribute to the contamination, growth, survival and dissemination of the suspected agent, prevention of future outbreaks and strengthening of food safety policies and programmes is not possible. Hence a regular monitoring and surveillance system like European countries in needed to combat foodborne diarrhoeal diseases.

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Comparative Histopathological Changes in Acute *Toxoplasma gondii* (RH Strain) Infected and Treated Mice

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Abstract

The aim of the present study was to investigate the comparative histopathological changes due to acute *Toxoplasma gondii* (RH strain) infection in infected and treated mice which might be useful observations for future research on toxoplasmosis in veterinary and medical science. Thirty Swiss albino mice were randomly divided into five equal groups (*i.e.* Gr. I, II, III, IV and V). The mice of group II, III, IV and V were intraperitoneally inoculated with 5×10^3 tachyzoites of serially passaged *T. gondii,* respectively). On three days post-infection (DPI) all the mice of group III, IV and V, showing the sign of acute toxoplasmosis (Ascites and respiratory distress) were treated with sulphadoxine with pyrimethamine @21.20 mg kg^{-1} body weight orally, azithromycin @200 mg kg^{-1} body weight orally and clindamycin @6 mg per mice, orally, respectively. Histopathological changes of spleen, liver, lungs and kidney of infected and treated groups were more or less similar. Spleen of Gr II, Gr III, Gr IV and Gr V revealed diffused infiltration of lymphocytes in the red pulp. Fatty degenerative changes in liver were the prominent feature in all the infected groups of mice. Lungs showed pneumonic changes and kidney showed intravascular haemolysis. Intestine of Gr III revealed perivascular cuffing of lymphocytes in submucosa and heart showed focal haemorrhages in myocardium whereas, intestine and heart of other treated groups did not reveal any significant changes.

Key words: Mice, *Toxoplasma gondii* (RH strain), Sulphadoxine-Pyrimethamine, Azithromycin, Clindamycin, Histopathology

Introduction:

Toxoplasma gondii is an obligatory, heterogenous, intracellular protozoan parasite which infects all warm blooded animals (Tenter et al., 2000, Dubey, 2009) including man and birds as intermediate host and cat as the definitive as well as intermediate host worldwide. It poses a great hazard to all immuno compromised hosts. It is usually asymptomatic in immune-competent individuals but may occasionally lead to severe ocular and neurological disorders (Furtado et al., 2013). When it comes to immune-compromised and congenitally infected individuals, toxoplasmosis can result in lethal systemic disease and eventually death (Shen et al., 2016). Currently, the control of *T. gondii* mainly depends on chemotherapy, such as the combination of sulfadiazine and pyrimethamine, but these drugs have serious side effects (Petersen and Schm, 2003). There are no effective drugs available to kill *T. gondii* cysts in tissues. Therefore, new efficient drugs and safe protective therapies are needed. Although spiramycin, clindamycin, azithromycin and sulphonamides in general are antitoxoplasmic.

There are several advantages of azithromycin over the more commonly used macrolides antibiotics, such as erythromycin. It has been reported that azithromycin in a 3-day course was found to be as effective as 5- to 10-day course of other antibiotics, including erythromycin, amoxycillin/calvulanic acid and penicillin V in treating respiratory tract infections (Dunn and Barradell, 1996). Oral treatment of this drug often associated with various adverse effects related to the gastrointestinal tract like cramping, diarrhoea, nausea, abdominal pain and vomiting (Dunn and Barradell, 1996). The reasons for the discrepant findings reported in animal models presumably include different experimental conditions comprising differences in both the infection model and treatment protocol. In this study, we screened three medicines such as Sulphadoxine-Pyrimethamine, Azithromycin and Clindamycin, that have different pharmacological effects and combined them .We evaluated the protective efficacy against the challenge of RH strain of *T. gondii* in a mouse model and to investigate the histopathological changes in different visceral organs.

Materials and Methods:

Mice: Thirty adult healthy Swiss albino mice of either sex aged 6-12 weeks and average weight of 25-30 gm were housed six per cage, and offered drinking water and feed *ad libitum* throughout the study period.

Parasite: The virulent modified mouse adopted RH strain of *T. gondii*, obtained from Division of Parasitology, Indian Veterinary Research Institute (IVRI), Izatnagar. – 243122, Bareilly, U. P, was maintained in our laboratory by continuous passage at 3-4 days interval in mice to produce (Sreekumar, 2001; Velmurugan, 2006). Peritoneal exudates of mice developed acute toxoplasmosis were cryopreserved for further study.

Grouping and experimental infection of the mice: The mice were randomly divided into five equal groups (*i.e.* I, II, III, IV and V). The mice of Group II, III, IV and V were experimentally inoculated with $5{\times}10^3$ *T. gondii* serially passaged tachyzoites intraperitoneally (I/P), and mice of Group-I were maintained as uninfected control.

Chemotherapy: The infected mice of group III, IV and V were treated with the following drugs when infection was noticed at 72-74 hours post infection (3rd DPI). The following drugs were used: On 3 days post-infection (DPI) all the mice of Gr III, IV and V, showing the sign of acute toxoplasmosis were treated with sulphadoxine with pyrimethamine @ 21.20 mg kg^{-1} body weight orally, azithromycin @ 200 mg kg^{-1} body weight orally and clindamycin @ 6 mg per mice, orally, respectively.

Combination of Sulphadoxine and Pyrimethamine: The *Toxoplasma* tachyzoites inoculated mice of Group-III having six mice were treated with sulphadoxine with pyrimethamine (Pyralfin, LupinPharma. Ltd.) @ 21.20 mg per kg body weight orally. Required amount of medicines were administered with PBS (pH-7.2). Ultimately 1ml of PBS (7.2) containing required dose of drug was given to each mice.

Azithromycin: The *Toxoplasma* tachyzoites inoculated mice of Group IV were treated with azithromycin (Azithral, Alembic Pharma Ltd.) @ 200 mg/kg body weight, orally as per Araujo et al. (1988).

Clindamycin: The *Toxoplasma* tachyzoites inoculated mice of Group V were treated with Clindamycin (Clincin, Indi Pharma Pvt. Ltd.) @ 6 mg per mice, orally as per Araujo et al. (1974). Required amount of medicines were administered with PBS (pH-7.2). Ultimately 1ml of PBS (pH 7.2) containing required dose of drug was administered orally to each mice.

Collection and staining of different organs: Mice of infected groups (Gr I and Gr II) were examined carefully and after confirmation of clinical signs, the mice were euthenized with diethyl ether or chloroform and post mortem examination was carried out. Then different organs like liver, lung, spleen, kidney, heart, intestine and brain were collected asceptically for histopathological examination. A piece of these organs were preserved in 10% formalin solution and the sections were cut and stained with Harris Haematoxylin and Eosin stain (H & E) following the standard method (Lillie and Fullmer, 1976).

Results and Discussion:

Histopathological changes:

Infected control group:

(a) Spleen: Diffuse proliferation of follicular lymphocytes obliterating the reticular structure of red pulp was noticed. Intravascular haemolysis was also found (Figure 1). Focal sub- capsular proliferation of reticuloendothelial cells along with lymphocytic infiltration with a few plasma cells was also observed (Figure 2).

(b) Liver: Intra and interlobular blood vessels of liver revealed intravascular haemolysis. Diffuse fatty changes were the prominent feature of liver hepatocytes (Figure 3). Focal precipitation of haemosiderin pigments were also noticed in the liver parenchyma.

(c) Lungs: Lungs showed focal areas of edema, pneumonia and emphysema. The pneumonic lesions comprised of neutrophils, lymphocytes with a few macrophages and plasma cells. Exfoliations of alveolar epithelial cells were also marked. Blood vessels of lungs also showed intravascular haemolysis (Figure 4 and 5).

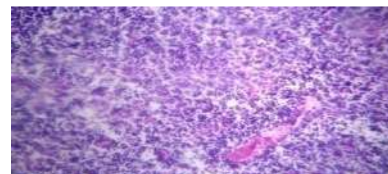

Figure 1: C. S. of spleen of infected mice showing diffuse infiltration of lymphocytes in the red pulp (H & E, ×400).

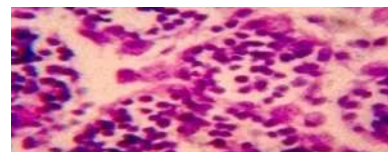

Figure 2: C. S. of spleen of infected mice showing diffuse infiltration of lymphocytes with subcapsular proliferation of endothelial cells (H & E, ×400).

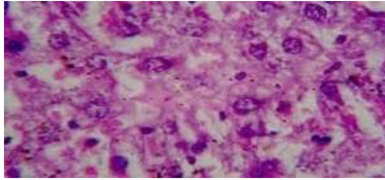

Figure 3: C. S. of liver of infected mice showing diffuse fatty degeneration of hepatocytes (H & E, ×1000)

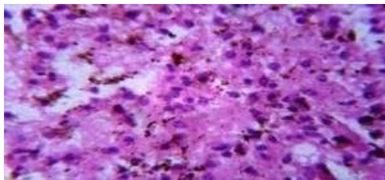

Figure 4: C. S. of lungs of infected mice showing focal area of edematous and pneumonic lesions with haemosiderin like pigments in lungs parenchyma (H & E, ×1000).

Figure 5: C. S. of lungs of infected mice showing congested blood vessels and emphysema (H & E, ×400).

(d) Kidney: Blood vessels of cortical and medullary regions of kidney were highly congested. Intravascular haemolysis and pigments of haemosiderin was also a common feature (Figure 6). Fatty and hyaline degenerations of the epithelium of proximal and distal tubules were also noticed.

(e) Intestine: Focal perivascular cuffing of lymphocytes in the submucosa was noticed. Diffuse infiltration of leucocytes particularly lymphocytes, neutrophils with a few macrophages and eosinophils in the villi of mucosa were also noticed.

(f) Heart: blood in the ventricle revealed massive haemolysis of red blood cells (RBC) with deposition of haemosiderin pigments. Focal haemorrhages in the myocardium were also evident (Figure 7).

Sulphadoxine and Pyrimithamine treated group:

(a) Spleen: Hyperplasia of follicular lymphocytes infiltrating the red pulp was the remarkable features.

(b) Liver: Diffuse fatty changes of the liver parenchyma were the prominent feature. Blood vessels of liver parenchyma showed intravascular haemolysis with haemosiderin pigments (Figure 8). Fibroblastic proliferation of interlobular septa was also noticed. Focal subcapsular fibroblastic proliferation with infiltration of lymphocytes, neutrophils and a few plasma cells were also observed.

(c) Lungs: Lungs was emphysematous and interalveolar septa were infiltrated by lymphocytes. Congested blood vessels showed partial intravascular haemolysis with sparse haemosiderin pigments.

(d) Kidney: Cortex showed the few atrophic glomeruli. Focal hyaline degeneration of both proximal and distal tubular epithelium with congestion of blood vessels both in cortex and medulla were noticeable features (Figure 9). Focal infiltrations of lymphocytes in the interstitial spaces of proximal and distal tubules were also observed.

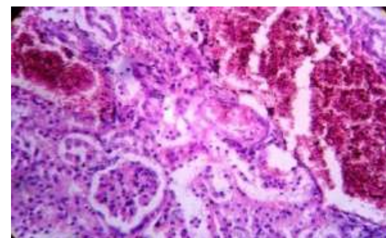

Figure 6: C. S. of kidney of infected mice showing intravascular haemolysis and haemosiderin like pigments in the cortex (H & E, ×400).

Figure 7: C. S. of heart of infected mice showing haemolysis with haemosiderin like pigments in the ventricle (H & E, ×100).

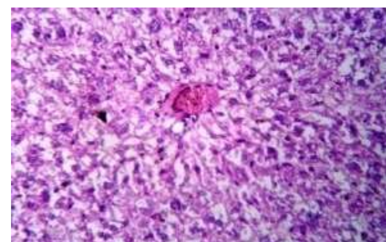

Figure 8: C. S. of liver of sulphadoxine-pyrimethamine treated mice showing diffuse fatty infiltration of the liver hepatocytes with intravascular haemolysis (H & E, ×400).

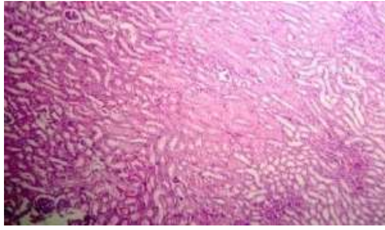

Figure 9: C. S. of kidney of sulphadoxine-pyrimethamine treated mice showing congestion of blood vessels in the cortex and hyaline degeneration of distal tubules (H & E, ×100).

Azithromycin treated group of mice:

(a) Spleen: Diffuse lymphocytic hyperplasia infiltrating the red pulp was the prominent features. Congestion of spleenic vessels with intravascular haemolysis was also noticed.

(b) Liver: Hepatocytes revealed mild fatty degenerative changes. Intravascular haemolysis in both inter and intralobular veins were prominent.

(c) Lungs: Focal edema and pneumonic lesions with lymphocytes, macrophages and a few plasma cells was noticed. Congestion of pulmonary veins with partial intravascular haemolysis with haemosiderin pigments were also revealed (Figure 10).

(d) Kidney: Intravascular haemolysis with pigments of haemosiderin found in the cortical region. Proximal tubular epithelium revealed focal hyaline degeneration (Figure 11).

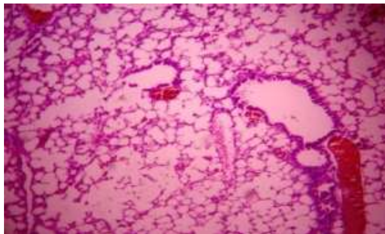

Figure 10: C. S. of lungs of azithromycin treated mice showing severe congestion of blood vessels with emphysema (H & E, ×400).

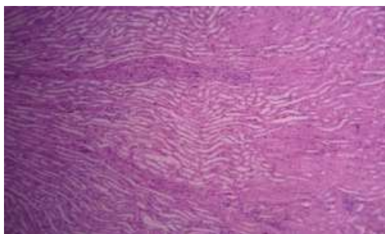

Figure 11: C. S. of Kidney of azithromycin treated mice showing hyaline degeneration of proximal tubules and focal interstitial cell proliferation in the medullary tubules (H & E, ×400).

Clindamycin treated group:

(a) Spleen: Follicular hyperplasia of lymphocytes with congested blood vessels was also noticed. Partial intravascular haemolysiswas the differentiating features.

(b) Liver: Diffuse fatty changes in the hepatocytes were the prominent features.

(c) Lungs: Lungs showed patchy areas of emphysema and pneumonia. Infiltrated cells in the pneumonic lesion were lymphocytes, neutrophils and a few plasma cells. Interalveolar septal edema is also present.

(d) Kidney: The epithelial cells of the both proximal and distal tubules were undergone hyaline degeneration. Blood vessels of the nephron around congested with intravascular haemolysis. Focal perivascular cuffing of lymphocytes with a few plasma cells were also noticed (Figure 12).

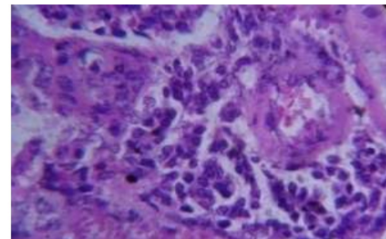

Figure 12: C. S. of Kidney of clindamycin treated mice showing perivascular cuffing of lymphocytes, mononucleate cells and few plasma cells (H & E, ×1000)

Diffuse proliferation of lymphocytes disrupting the follicular nature of white pulp, with intravascular haemolysis of RBC in the spleen of Gr II and Gr IV were the prominent feature (Figure 1 and 2), which indicated the infection was of acute in nature. Whereas, mice of Gr III and Gr IV showed follicular lymphoid hyperplasia of white pulp of the spleen, which was indicative of subacute type of infection. This was in agreement of findings of Suzuki et al. (1973) and Rifaat et al. (1981). A supra-capsular cyst (Figure 12) was found in one mice of Gr-II. The cyst was full of caseated mass. Subscapular proliferation of reticuloendothelial cells along with lymphocytic infiltration with a few plasma cells was also observed the same focal area. Such type of cyst might be produced due to trauma of needle ordue to liberated toxinsby the attached tachyzoites to form such caseated cyst in supracapsular region of spleen.

Diffuse fatty changes in the liver of the infected group (Gr II) and treated groups (Gr III, Gr IV and Gr V), might be due to toxins produced by the tachyzoites. Grimwood et al. (1983) also mentioned the same and named Toxofactor, a glycoprotein molecule (Mw 50-100 kDa)

respectively for such type of changes. All the blood vessels of spleen, liver, kidney and lungs showed intravascular haemolysis of Gr II, Gr III and Gr IV, except Gr V which showed partial intravascular haemolysis.

The same toxic factor might be responsible for intravascular haemolysis in the organs, which reflected erythrocytopenia and leucocytopenia (Sthal et al*.,* 1998). The Gr V mice treated with clindamycin, shown partial intravascular haemolysis. Clindamycin was moderately effective with its static effect on both extra and intracellular tachyzoites in macrophages, thus hindered the release of toxins from tachyzoites phagocytosed (Filice et al*.,* 1991).

Excretion of toxic metabolites through kidney inflicted hyaline degeneration of proximal and distal tubules and congestion of blood vessels both in cortex and medulla (Figure 19). The similar results also reported by Shalaby et al. (1993) that was revealed in Gr II, Gr III, Gr IV and Gr V mice in the experiment. Lungs of infected and group (Gr II) and treated groups (Gr III, Gr IV and Gr V) of mice had showed focal pneumonia and oedema. Emphysema developed as a sequele of same. Davidson et al*.* (1996) also focused that generalized toxoplasmosis as increased morbidity and mortality occurred from hepatitis and pneumonia. Mice treated with clindamycin (Gr V) had shown focal leucocytic infiltration in the villi and hyper activity of goblet cells in the intestine, which might be due to local infiltration of the drug during absorption through intestine.

Conclusion:

Since the mice are the most studied models for experimental toxoplasmosis the findings of the present study should be useful observation for future research on either acute toxoplasmosis in animal or human, or disseminated toxoplasmosis in AIDS patients.

Conflict of interest:

Authors have no conflict of interests in this study.

Data availability:

All the data in relation to the present study are available.

Author's contribution:

All authors equally participated in designing this article, data analysis and interpreting the results, drafting, editing the manuscript and approved the final version of the manuscript.

Ethical approval:

Obtained from the Institutional Animal Ethical Committee of WBUAFS vide reference no. E. Com. 55 dated 15.06.2010.

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Original Research Article

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Experimental Pathogenicity of *Papiliotrema laurentii* in Swiss Albino Mice

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Abstract

Cryptococcus may be found as pathogenic fungi which have rarely been studied. In this study the virulence factor of non-neoformans *Cryptococcus* species, biological characteristics of the fungal pathogen and pathophysiology of mice infected with the Cryptococcal species *Papiliotrema laurentii* compared with normal mice for definite time intervals. All the test mice were inoculated with the yeast cells of cryptococcal organisms that showed an almost uniform pattern of behavior in relation to the isolation of the same specific organisms from different organs of the infected mice during the interval of the experimental period. Tissue samples were studied after processing from the both dead and also from the mice scarified on the scheduled day of post infection except from those of the control group. The yeast cells were cultured following standard protocol and the biological characteristics were also studied. The pathogenicity of non-neoformans species of the genus did not show much pathogenicity in mice. The findings of the present study also indicated that the cultural and biochemical characteristics and, also pathophysiology of the species *Papiliotrema laurenti* in mice. However, No recovery of *Papiliotrema laurentii* was observed from any organs on this specific day of post infection with this organism *i.e. Papiliotrema laurentii* which made the study a significant observation. The pathogenic effects of this species were not the same in different time intervals. This reflects that virulence factors are related to time intervals in mice infection regimens before isolation from organs. Histopathological findings also were found from liver and brain. As Cryptococcosis a zoonotic disease, study of virulence factors of the organisms in mice modal may help in understanding the transmission of this disease and its pathogenicity.

Keywords: *Papiliotrema laurentii, Swiss albino mice,* Experimental infection*,* Virulence factors.

Introduction:

Cryptococcosis continues to exacerbate as the systemic and opportunistic mycoses, with potential/severe health risks, especially in high-risk groups and immunocompromised patients. *Cryptococcus neoformans* is regarded as the major human and animal pathogen, with *Cryptococcus laurentii* to be occasionally known to cause moderate to severe diseases, especially in immune-compromised patients (Cheng et al., 2001).

The zoonotic importance and epidemiology of *Cryptococcus neoformans* have been reported frequently, yet there is lack of proper information as literature are scanty on the non-neoformans species like *Cryptococcus albidus, Cryptococcus laurentii*or *Cryptococcus uniguttulatus* (Rosario et al., 2008). They also reported that crops and droppings of pigeon loft and other environmental sources including canopy leftovers from some trees are regarded as the main sources of *Cryptococcus laurentii,* the distribution that lead to infection in human and animals (Bernardo et al., 2001; Jang et al., 2011; Kamari et al., 2017).

The epidemiology study of non-neoformans *Cryptococcus laurentii,* and some other species of the genus are highly relevant as these species often turn out to be pathogenic and thereby increase the risk of infection. A case of pulmonary cryptococcosis due to *Cryptococcus laurentii* in a diabetic patient and patients suffering from ganglio-neuroblastoma was also reported by Averbuch et al. (2002) and Shankar et al. (2006).

In this background, the *in vivo* pathogenicity study in appropriate animal models is highly useful in establishing the pathogenesis and pathology of disease caused by microorganism that can be helpful to observe the efficacy of drugs against the pathogenic microorganism. So, the experimental pathogenicity study in Swiss albino mice was selected with *Papiliotrema laurentii* to study in details all about this opportunistic pathogen (Sukroongreung et al. 1999; Chander, 2002). The histopathological techniques were also used in this

retrospective studies, to delineate the infectivity of the tested isolates were also cross checked (De Bernardis et al., 1987). Here the experimental pathogenicity of non-neoformans *Cryptococcus* species was compared with normal mice in a definite time interval to gain a definite response.

Materials and Methods:

In vivo pathogenicity study:

***Fungal test* strain:** Cultures of *Papiliotrem alaurentii* (Gene Bank Accession number MT102623) isolated in the department of Biotechnology Gauhati University conducted during the research works done by the authors (Islam et al., 2020) were used in the present studyfor testing the pathogenicity and conducting histopathological studies in Swiss albino mice.

Reference strain: *Cryptococcus laurentii* (MTCC 2898) procured from CSIR IMTECH, India was used as reference strain in this study.

Experimental animals:

A total of fifty (50) disease-free Swiss albino female mice of 5-6 weeks old, weighing 22 -25g was gifted by Prof. C.C. Barua, Department of Pharmacology and Toxicology, College of Veterinary Science, AAU, Khanapara, Guwahati, which were used for the study. The mice were maintained in animal house as laboratory animals of the Department of Biotechnology, Gauhati University and were provided with *ad libitum* drinking water and formulated feed as per requirements. Protocols for the experiments on laboratory animals were performed in accordance with the approval of the Institutional Animal Ethical Committee of Gauhati University vide reference no. AEC/PER/2019/BBC/AS/2019-030 dated. 08/05/2019.

Experimental design:

Inocula/ Culture of *Cryptococcus* Species:

Cultures of MT102623 (Isolated in the Department of Biotechnology, Gauhati University, Assam, India), and reference strain MTCC 2898 maintained on SDA at 37°C were transferred into tubes containing 20 ml of BHI (Brain Heart Infusion) broth and then kept at 30°C. Inocula were prepared as per De Bernardis et al. (1987).

Animal study and infectious challenge:

Design for experimental infection in animals was done as was described by Banerjee et al. (2017), Pedroso et al. (2009), Known Chung et al. (1992) and Nishimura and Miyaji (1979) with slight modifications.
After acclimatizing the experimental animals for about one week, animals were assigned into four groups: GroupI and Gr II consisted of 13 numbers of mice each, injected with infective inocula of *Papiliotrema laurentii* (MT102623) and reference sample *Papiliotrema laurentii* (MTCC 2898) respectively. The last group (Group III) comprised of 10 numbers of mice that were used as controls and injected only with the sterile PBS. Each mouse in the experimental Groups (I, II) were challenged with the 100 µl cell suspension of the specific inocula of tested yeast cells (1 x 10^7 cells) as mentioned in Table 1. All the mice in the control group (Group III) received sterile PBS injections.
Mice were examined twice in a day for recurrence of any diseased symptoms and progression or potential side effects *i.e.* laboured breathing, incoordination of gait, weight loss, edema, congestion of meninges, ruffling of furs etc. During experimental period, mortality of mice, if any, before or after the assigned days up to 28 days were recorded.

In vivo pathogenicity study and histopathology:

All the experimental mice were euthanized with ketamine @ 200 mg/kg body weight (bwt) intravenously on the 2nd, 7th, 14th, 21st, and on the 28th day of post-infection with inocula of *Cryptococcus* considering the day of injection with the inocula as 0 day and of postmortem changes in liver, brain, lungs, kidneys etc. were also recorded and histopathological examination of different organs of the animals challenged with the inocula of *Cryptococcus* were performed. During the experiment, mortality assessment of the animals *in vivo* was also done. However, our study was mainly restricted to histopathological examination of liver and brain only.
At necropsy, liver, lungs, kidney and brain tissues were removed aseptically both from sacrificed as well as from dead mice, if any, immediately on the day of sacrifice as mentioned in the Table 1, and the collected specimens of were used to determine fungal loads as well as other morphological and biochemical studies. The liver and brain tissue samples stored separately in containers containing 10% buffered formalin for histopathological studies. After paraffin inclusion of the slices of the organs were dehydrated and tissues were processed employing conventional procedure and were cut into 4-5 µm thick sections stained with hematoxylin and eosin (H&E) (Culling et al., 1963; Luna et al., 1968; Suvarna et al., 2012) with slight modifications, and observed for morphological changes. The dry mounted slides were examined for interpretation.

Quantitative assessment of *Cryptococcus* in the infected areas:

Degree of quantitative assessment of fungal burden in brain and liver in 10 different selected fields were observed randomly at 400x magnification. Absence of no fungi in tissues was marked as (0), area of fungal infection in the field ≤ 25 % was marked as(+), infected

area with 25 % to 50 % was marked as (++)whilst the areas with ≤ 50 % infection was marked as (+++) (Surawut et al., 2017).

Culture for recovery of *Cryptococcus* species and *in vitro* pathogenicity:

Specimens from various organs *i.e.* liver, lungs, kidney, spleen, and brain of the challenged mice were processed in appropriate SDA media. Cultural and biochemical characteristics of the yeast cells were performed following the procedures and maintaining standard protocol.

Growth at 37°C:

For recovery of the test organisms the cut segments of different organs of the experimental animals, were homogenized separately in 10 ml of PBS. 100µl of the homogenates were plated on to SDA media and were incubated at 37°C for 48 hours following the procedure as described by Pal (1997c) and Rosario et al. (2008) with slight modifications.

Urease activity:

A loopful of test culture of *Cryptococcus* was inoculated onto urease agar slants. The slants were monitored for every 6 hours interval to observe change of colour up to 2 weeks. A positive reaction was indicated by change of colour from yellow to pink or red. Absence of change in colour was considered as negative. The test was performed following the procedures of Christensen (1946).

Nitrate reduction test:

This test was done for differentiation of the member of the *Cryptococcus* species on the basis of their ability to reduce nitrate to nitrite following the procedure of Rhodes and Roberts (1975). Inocula of the test organism were incubated in nitrate broth. After 4 hours of incubation the broth was tested for reduction of nitrate to nitrite by adding sulfanilic acid reagent and ∞-naphthylamine. Change of colour to red was considered as positive for the test.

Carbohydrate assimilation Test:

Media for carbohydrate assimilation was prepared by adding 0.675 g of yeast nitrogen base (YNB- Himedia) and 2 mg of bromocresol purple (Himedia) with 100 ml of deionized water. The media was autoclaved at 121°C for 15 minutes, cooled to room temperature and then 10 ml of media was poured in test tubes containing sugar discs of 25 mg (Himedia) that contained glucose, lactose, maltose, trehalose, sucrose and lactose. The media was then inoculated with test isolates and incubated at 30°C and absorption maxima were recorded daily up to 96 hours. Change of colour from purple to yellow indicated a positive result. The test was conducted as was described by Chakrabarti et al. (1998).

Carbohydrate fermentation Test:

This test was performed with peptone water in phenol red (Himedia) as was described by MacFaddin (1985). The media after autoclaving was inoculated with loopful of cryptococcal cultures and subjected to Durhams tubes containing 5 ml of peptone water and the carbohydrate discs of glucose, lactose, maltose, trehalose, sucrose and lactose. The contents were incubated at 28°C for 28 to 48 hours. Presence or absence of gas in the tube was indicative of a positive or negative result.

India ink staining for capsule identification:

For capsule identification, fresh fungal inoculum prepared in SDA was used whereby one drop of suspension was added in India ink on a sterile glass slide and covered with a cover slide and observed microscopically in 10X and 40X magnifications (Kwon-Chung and Bennett, 1992). The slides were also subjected to phase contrast microscopy under 100X magnification. Presence of distinct wide gelatinous capsules, round to oval with or without hyphae was considered as positive.

Results and Discussion:

In the past few decades, increase of incidence of infections with non-neoformans species *Cryptococcus laurentii* have been reported and both the fungal organisms cause about 80 percent of the related non-neoformans and non-gattii Cryptococcal cases (Khawcharoenporn et al., 2007). Many works have been reported on the zoonotic importance and epidemiology of *Cryptococcus neoformans*, but literatures are scanty on the occurrence, distribution, epidemiology and surveillance studies on the non-neoformans fungal pathogens, *Cryptococcus albidus, Cryptococcus laurentii* and other species. Environmental isolates are regarded as the main sources of *Cryptoccus albidus*, *Cryptoccus laurentii* and *Cryptococcus uniguttulatus* that lead to the transmission of infection in human as well as animals (Bernardo et al., 2001; Jang et al., 2011; Kamari et al., 2017). *Cryptococcus laurentii* is currently characterized as a human pathogen and is responsible for causing infection exclusively in immune compromised individuals (Averbuch et al., 2002). Studies on the *Cryptococcus albidus* and *Cryptococcus laurentii* in experimental animals, mainly in immunocompromised animals have rarely been studied (De Bernardis et al, 1987).

***In vivo* pathogenicity study:**

Clinical observations of infected mice:

In the present investigation, all the treated mice were observed for 28 days of post fungal treatment to observe

Table 1: Experimental design of animal study with the infective inocula of *Cryptococcus* organisms										
Sl. No	Group	Inoculated with	Dose of inocula (Cells)	Day of Inoculation	Nos. of mice sacrificed and days at					No. of mice
					2	7	14	21	28	
1	I	Infective inocula of MT 102623	1 x 10^7	0 day	1	3	3	3	3	13
2	II	Infective inocula of MTCC 2898 (ref. sample)	1 x 10^7	0 day	1	3	3	3	3	13
3	III	Sterile PBS	-	0 day	2	2	2	2	2	10

Table 2: Mortality of mice after fungal infection				
Group	No. of mice died up to the day post infection	Survived upto 28th day	Cumulative mortality	% of death
I	1 (on 19th day	5	1	20.00
II	1 (14th day)	6	1	16.67
Total	**2**	**11**	**2**	**18.19**

the presence of any clinical signs of infection or any death due to infection.

It was observed that few numbers of mice showed more or less clinical signs on different days of post infection. As presented Figure 1, two numbers of mice depicted enlargement of lymph glands on 28th days of post-infection with *Papiliotrema laurentii*. Two numbers of mice died on the 14th and 19th days of post treatment (Table 2) that showed the clinical features of reduced feeding and water intake representing dehydration and emaciation **(**Figure 2**)**. All other mice in all the 3 groups (I, II, and III) during the follow-up period up 28 days of post treatment were found to be healthy as no clinical features or symptoms of diseases could be observed except the two mice died on the 14th and 19th day of post treatment given with the organism.

Cultural, morphological and biochemical tests of the isolated strains represent similar patterns of reaction as of those inoculated strains of *Papiliotrema laurentii* introduced into experimental mice. The results are comparable with the reference strain MTCC 2898.

Figure 1: Enlargement of superficial cervical lymph node at 28th day of post infectionby *Papiliotrema laurentii*

Clinical and pathological findings of the infected mice:

After sacrificing the mice as mentioned in materials and methods, necropsy was performed to observe the pathological changes in brain, liver, kidney, lungs and other organs. In the study, no gross lesions were observed in different organs upto one week of post treatment. The mice that died during 14th and 19th day of post infection appeared dehydrated and emaciated with congestion of the brain and lungs, swollen kidneys and hemorrhages in the liver and brain and the symptoms were similar with the mice that were sacrificed as on the predetermined dates (Figure 3). Mice in the control group during the entire experimental period remained healthy and no abnormality could be detected during post mortem examination.

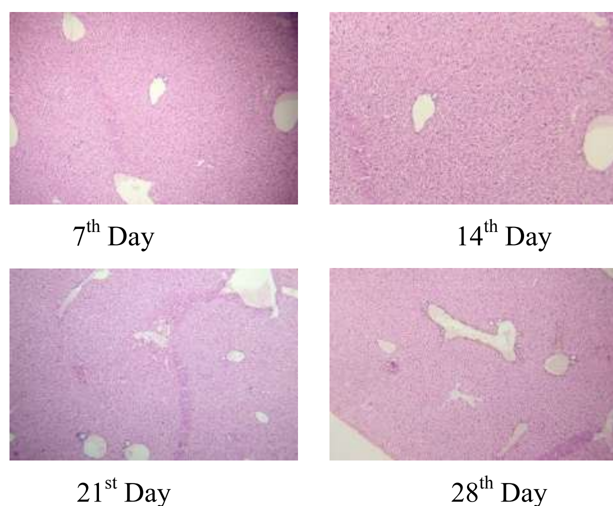
Figure 2: Haemorrhages in brain tissues on the 21st day of post infection with *Papiliotrema laurentii*

Figure 3: Haemorrhages in liver tissues on the 21st day of post infection with *Papiliotrema laurentii*

Recovery of *Cryptococcus*, cultural characteristics and *in-vitro* pathogenicity test:

Growth at 37°C

The specimens collected from the infected animals after sacrifice from Groups I, II and the control group III were processed and streaked on SDA plates in duplicates, incubated at 37°C for 48 hours. Fungal organisms were further isolated from specimens of different organs *ie.* specimens of different organs of mice I, II, and but no isolate of the fungal organism of *Cryptococcus* could be done from the specimens of Grp. III (Figure 4).



7th Day 14th Day

21st Day 28th Day

Figure 4: Liver tissues on control mice observed through 40X magnification.

Histopathological degree of quantitative assessment of post infection of *Cryptococcus* organisms in liver and brain after sacrifice of infected mice indicates highest degree of clinical manifestation and damage in liver at 28 days (+III level) and in brain at 21 and 28 days (+II level). The results are presented in Table 3.

The present investigation showed experimental pathogenicity of *Papiliotrema laurentii* in definite time interval. The species were investigated in relation to the pathogenicity and histopathological changes, especially in liver and brain of mice infected with inocula of the organisms under the study. The study revealed that biological characteristics and pathophysiology of infected mice to non-neoformans species of Cryptococcus are almost similar with *Cryptococcus neoformans* which emphasized the degree of virulence and level of pathogenicity to an extent comparable with the previous studies exhorted by other researchers.

Similar observations were also observed by Grosse et al. (1975) in experimental murine model inoculated *Cryptococcus* organism *Cryptococcus cereanus and Cryptococcus albidus*. De Bernardis et al. (1987) observed that the species of non-neoformans *Cryptococcus* species do not substantially differ from the pathogenic *Cryptococcus neoformans* in the mechanism of invasion and in the kind of host tissue damage, once the host's immune defense mechanism has been sufficiently impaired.

Capsule identification in India Ink and Phase contrast microscopy:

Capsules of both test and reference strains of tested fungi from specimens were distinct, wide, gelatinous, round, and oval without hyphae were observed under phase contrast microscopy (Figure 5, 6, 7).

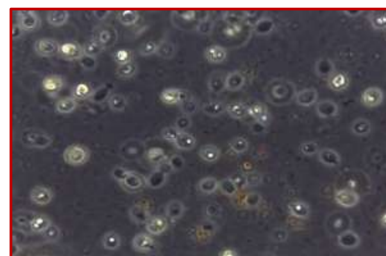


Figure 5: Phase contrast microscopy of liver tissues of albino mice infected with *Papiliotrema laurentii* (observed on 21st day after treatment)

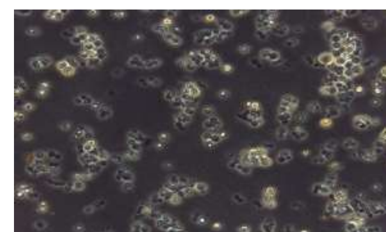


Figure 6: Phase contrast microscopy of liver tissues of albino mice infected with reference strain no. MTCC2898) (observed on 21st day after treatment)

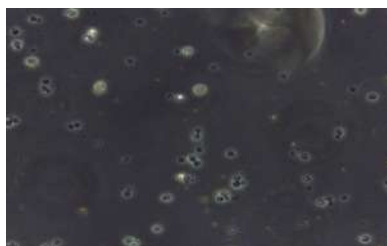


Figure 7: Phase contrast microscopy of brain tissues of albino mice infected with reference strain no. MTCC2898) (observed on 14th day after treatment)

Biochemical characterization:

In Urease production, nitrate reduction, Carbohydrate assimilation, Carbohydrate fermentation and Phenoloxidase test, the test samples show typical results. Isolates of both tests and reference strain of *Cryptococcus* (MTCC 2898 – *Papiliotrema laurentii*) were found positive for different sugars viz. glucose, maltose, sucrose, lactose, galactose and trehalose. Meanwhile, the fermentation test and phenol oxidase tests were found to be negative.

***In-vitro* pathogenicity test for the control group:**

All the tests, - growth at 37°C, capsule identification in India Ink, Urease production, Nitrate reduction test, Carbohydrate assimilation, Carbohydrate fermentation, and Phenol oxidase test were found negative.

Histopathological findings

Changes in liver:

Histopathological studies of the liver sections showed recovery of yeast cells 2 days of post infection with the test organisms *Cryptococcus* but from the 14th day of post infection onwards to the subsequent observations upto 28th days of experiment revealed that the cells of *Cryptococcus* spp. Significant changes could not be recorded in respect of yeast cells of *Papiliotrema laurentii.*

Yeast cells of *Cryptococcus* species, were distinctly observed in the section of the liver from 21st and 28 th day of post infection (Figure 8, 9). At the 21st day of post infection, sections of the liver revealed congestion, hemorrhages and coagulative necrosis. Blood vessels were highly congested and dilatation of sinusoidal spaces was observed. Fat-vacuoles were detected within the hepatocytes which pushed the nucleus to the periphery of the cells. Focal areas of coagulative necrosis were also detected throughout the hepatic parenchyma. Control Grp. mice show no such changes. Nishimura and Miyaji (1979) also observed high susceptibility among mice and reported that cell mediated immunity may play an important role as the defense mechanism against cryptococcosis and confer survivability in mice against cryptococcosis.

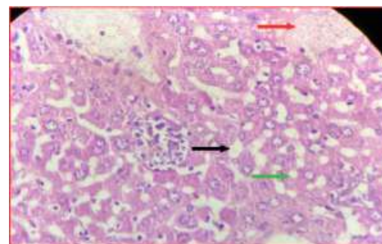


Figure 8: Changes in liver tissues of albino mice on the 21st day of post-infection with *Papiliotrema laurentii* showing congested blood vessels (red arrow), fatty change (green arrow), and dilatation of sinusoidal spaces (black arrow) (HXE, 40X)

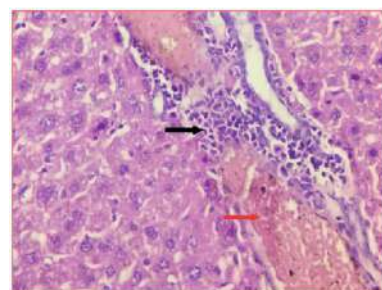


Figure 9: Changes in liver tissues on the 28th day of post-infection with *Papiliotrema laurentii* showing massive aggregation of fungal cells surrounding the congested blood vessels (red arrow) and coagulative necrosis (black arrow) (H&E, 40X)

Changes in the brain

Considerable histopathological changes in the brain sections of the infected mice of all the experimental groups were observed during the study. No significant changes were noticed with this fungus. Cerebellum of mice at the 14th day of post treatment by *Papiliotrema laurentii* showed demyelination (red arrow) and satellitosis (Figure 10).

Histopathological changes of the brain tissues of the infected mice sacrificed on the 14st day of post infection revealed degeneration of purkinjee cells in the cerebellum. Presence of cysts of the test fungus was noticed in the molecular layer of the cerebellum (Figure 11).

After 28 days of post infection, the brain sections revealed degeneration of purkinje cells of the cerebellum. A few fungal cysts were also observed in the granular layer of the cerebellum (Figure 12).

Gyu-Nam Park et al. (2018) reported the recovery of the *Cryptococcus* species complex associated with the severity of histopathological lesions which included

hemorrhage, inflammation, and tissue damage in different organs, especially in the lungs and liver of experimental rats. These results indicated that the pathophysiology of the *Cryptococcus* species complex infection differs according to different species and serotypes.

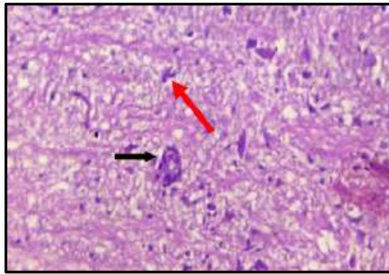


Figure 10: Changes in thecerebellumon the 14th day of post infection with *Papiliotrema laurentii* showing demyelination (red arrow) and satellitosis (black arrow) (H&E, 40X)

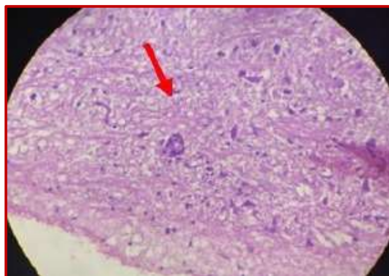


Figure 11: Cerebellum of mice at the 14th day of post infection by *Papiliotrema laurentii (reference strain)* showing demyelination (red arrow) and satellitosis (H&E, 10X)

Previous studies also confirmed that *Cryptococcus neoformans* strains (serotype A, D) and A/D were the main causative agents of fatal cryptococcal disease in rat (GyU-Nam Park et al., 2018). In another study, Thompson et al. (2012) also reported that the median survival in mice infected with *Cryptococcus neoformans* is 19.5 days in contrast to10.5 days in mice infected with *Cryptococcus gattii*.

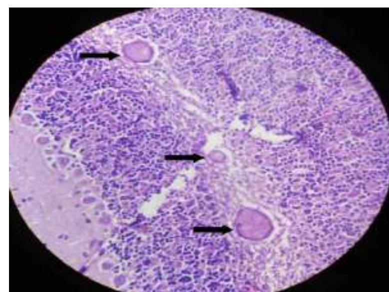


Figure 12: Changes in thecerebellum on the 28th day of post infection with *P. laurentii*showing appearence of the fungal cyst in the granular layer (black arrow) (H&E, 40X)

The observations with respect to the virulence of *Papiliotrema laurentii* were found similar with the studies reported by Pedroso et al. (2009a), De Bernardis et al. (1987), Fellandstatzell – Tallman (1998), and Grosse et al. (1975). Capacity of the tested *Cryptococcus* species suggested the environmental acclimatization of these pathogenic yeasts into animals and cause infection which was also substantiated with the studies of Pedroso et al. (2009a, 2009b). It is generally known that the major infection sites of the *Cryptococcus* species are complex in brain and lung tissues. These fungal pathogens may spread haematogenously to other organ systems causing severe damage (Buchanan and Murphy, 1998; Lortholary et al., 1999; Hansong and Robin, 2009; Negroni, 2012). Okubo et al. (2013) also reported on histopathological findings of the lungs between mice infected with *Cryptococcus gattii* and *Cryptococcus neoformans* and differences in pathophysiology that led to the death of mice.

Tissue sections of the brain in the control group:

Histopathological sections of brain tissues appeared normal. Neurons and the glial cells appeared intact. In the cerebellum, the granular as well as molecular layers appeared normal. Purkinje cell layers were also observed to be intact (Figure 13).

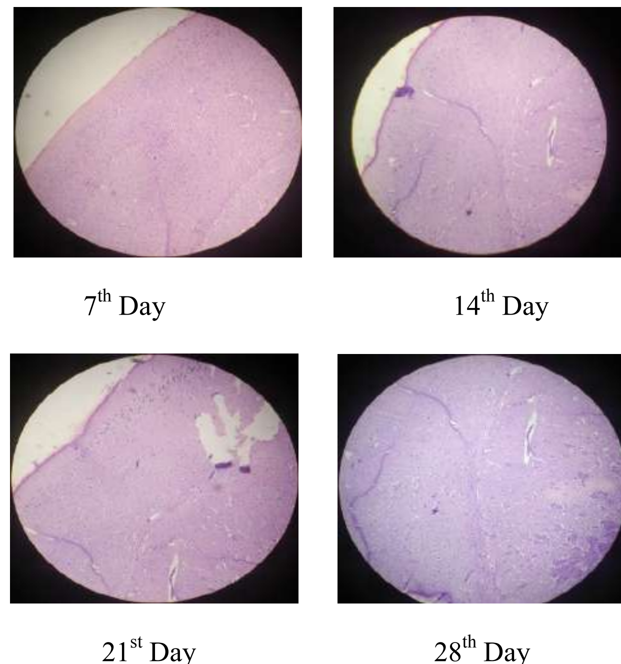


Figure 13: Control sample of Brain tissues

Table 3: Histopathological quantitative assessment of post-infection of *Cryptococcus* organisms in liver and brain after sacrifice of infected mice										
Group of experimental animals	**Post-infection (PI) date and quantitative assessment**									
	2 days		**7 days**		**14 days**		**21 days**		**28 days**	
	Liver	**Brain**	**Liver**	**Brain**	**Liver**	**Brain**	**Liver**	**Brain**	**Liver**	**Brain**
Group I	0	0	+I	+I	+I	+I	+II	+II	+III	+II
Group II	0	0	+I	+I	+I	+I	+II	+II	+III	+II
Group III (control)	---	---	---	---	---	---	---	---	---	---

Conclusion:

This study suggested that the pathogenicity of non-neoformans species *Papiliotrema laurentii* did not differ substantially with the other strains of *Crypotoccus neoformans* in respect of mechanisms of infection, invasion, host tissue damage and also the important role of cell mediated immunity. The findings of the present study also indicated that the cultural and biochemical characteristics and also pathophysiology of both the species. *Papiliotrema laurentii* in mice model did not substantially show many effects. Histopathological findings induced by the infection of this *Cryptococcus* species was essentially similar considering the target organs – liver and brain. It was reported that the absence of molecular alteration in the genotype of *Papiliotrema laurentii* and that the virulence factors of both the species were not related to time intervals of mice infected with the organisms and the subsequent effects due to post-infection. As cryptococcosisis a zoonotic disease, the current report thus may provide information on the pathophysiology and clinical observation of *Papiliotrema laurentii*as potential pathogenic fungal zoonosis.

Conflict of interest:

The authors declare that there is no conflict of interest.

Data Availability:

As per the research design, Gene Bank Accession Number NCBI data from (MT 102623) of the isolates of the original research of the authors and *Cryptococcus laurentii* (MTCC 2898) procured from CSIR IMTECH, India was used for the study.

Authors' Contributions:

All authors listed have made a substantial, direct, and intellectual contribution to the work.

Ethics Statement:

Obtained from the Institutional Animal Ethical Committee of Gauhati University vide reference no. AEC/PER/2019/BBC/AS/2019-030 dated. 08/05/2019.

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Original Research Article

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Safety Study Evaluation of Herbo-metallic Nanodrugs in Carcinogenesis Model

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Abstract

Since ancient times, mineral-based treatments have been frequently employed as anti-carcinogenic agents. However, due to a lack of safety research on the usage of this kinds of herbal drugs, caused hindrance in its wider applicability. Although they are proven to be anti-carcinogenic agent and could precisely be effective in cancer treatment. Rasa manikya and Rasa sindhura are herbo-metallic nanodrugs enriched with arsenic and mercurial product. These are anti-carcinogen used as a major mineral ingredient in traditional pharmaceutical science of rasa Shastra. To harness the application of rasa manikya and rasa sindhura the present investigation was taken up to assess the safety and efficacy of the drugs against skin cancer in Balb/C mice and data were obtained by functional observational battery (FOB), haematological and serum enzyme analysis from the experimental animal for 16 weeks observation period. Both the herbometallic drugs in their nanoform were proven to be effective as anti-carcinogenic agent and maximum efficacy dose for rasamanikya and rasa sindhura was found to be 10 mg/kg body weight in this experiment.

Key words: Herbo-metallic nano drug, Rasa manikya, Rasa sindhura, Safety study

Introduction:

A growing number of people are using herbal treatments since they are thought to have negligible or no negative effects. Eighty percent of people in nations like Asia and Africa receive their primary medical care from traditional practitioners. Among traditional medications, herbal remedies are the most profitable, bringing in billions of dollars annually (Rastogi et al., 2015). Approximately 5000 years have passed since the first uses of minerals and rocks for medicine. Together with plants and animal parts, minerals and rocks are also frequently used in the preparation of pharmaceuticals in Ayurvedic medicine, a well-known traditional indigenous medicine practiced in South Asia (Wijenayake et al., 2014).

Rasashastra is a branch of Ayurveda that is covered under the Indian Science of Alchemy which focuses on mineral medications, special processing methods, the therapeutic use of minerals, and their qualities (Sarkar and Choudhury, 2010). Several minerals have been utilised in ayurveda to make various therapeutic goods, including mica, realgar (As_4S_4), orpiment (As_2S_3), pyrite (FeS_2), chalcopyrite ($CuFeS_2$), magnetite (Fe_3O_4), hematite (Fe_2O_3), galena (PbS), salts (NaCl/KCl), chalk ($CaCO_3$), jewels, and clays (Mishra, 2004). Even today's pharmaceutical industry uses a wide array of minerals as active components. Certain metallic ions have a significant effect on altered cellular metabolism, which prevents cancer cells from proliferating and spreading (Ruidas et al., 2019).

Rasa Shastra is researched under several heads, such as Ratna-Upratna, Visha-Upavisha, Rasa- Maharasa, Uparasa, Sadharana rasa, and so on. The medications are categorised in these sections based on their characteristics, modes of occurrence, and utility for specific biological processes. One such medication that falls under the Uparasavargadravya category is haratala, which is harmful since it contains an arsenic ingredient. In several forms, Haratala is frequently employed such as shodhita, bhasma and rasamanikya. One of these is the light microfine powder known as Rasa Manikya, which is made by processing Patra Haratala (Sud, 2013) Rasasindura, a unique ayurvedic mercurial mixture, has also been used for a long time to cure a number of disorders, such as pyrexia, hepatic problems, sexual problems, neurological and immunological concerns (Kamath et al., 2012).Rich in antioxidants like vitamin E, sesamol, and sesaminol, sesame oil is also a herbal remedy that has been demonstrated to have potent anti-inflammatory and anti-diabetic properties. These properties can help with chronic inflammatory conditions like scrapes, joint inflammation, and diabetic neuropathy. (Ramesh et al., 2005).The purpose of the present study is to evaluate the safety in their therapeutic dose for a chronic period how far the effect of these compounds on long term therapy in biological system and how far the alteration in behaviour while active dose regimen is

scheduled to control the cancer using herbal oil and herbo-mineral medications by functional observation battery, serum enzyme analysis and haematological analysis.

Materials and Method:

Animals: The study had the previous permission from the Institutional Animal Ethics Committee (IAEC), which is supervised by CPCSEA, Government of India. The study approval number is ERS/IAEC/2022-23/INST/004. A total of 30 male Balb/C mice were of 6-8 weeks were housed in an open-cage facility under regulated conditions for animal experiments. According to the CPSCEA's recommendations, the temperature and relative humidity were kept at 20–26 °C and 30–70%, respectively. The bedding material was made of sterilized maize cobs. Cage and bedding materials were changed twice a week. Detergent was used to clean the cages, and hydrogen peroxide and pure alcohol were used for sanitization. Water and food (rodent pellet feed) were supplied ad libitum.

Experimental design: The safety mechanism of herbo-mineral medicines and fortified oil was examined using a two-stage skin cancer development model. The successive application of a sub threshold dosage of a carcinogen (initiatior), followed by repeated exposure to a non carcinogenic promoter (promoter), induces the hallmark of two-stage carcinogenesis. The animals were divided into six groups: positive control, negative control, rasa manikya, rasa sindhura, sesame oil, and imiquimod group. Depilatory cream was used to remove the hairs on the dorsal side at the base of the tail (3cm diameter). The depilated backs of the mice were sprayed with ethanol/acetone, before being coated with 0.1ml DMBA and 1% croton oil twice and thrice a week for up to 8 weeks, respectively. The first group kept as positive or disease control without any treatment and the second group as negative/healthy control without the induction of tumour. Third, fourth, fifth group were treated orally with rasa manikya (@10.16mg/kg), rasa sindhura (@10.16mg/kg), sesame oil (@5ml/kg) for 16 weeks and in the sixth group, imiquimod was applied topically after the formation of tumour (*i.e.* 12 weeks) at a concentration of 0.25 gm in 5ml of distilled water, 0.1ml on each mouse.

Functional Observation Battery (FOB): Functional Observation Battery evaluation was performed during 16 weeksperiod for all the groups at predose and postdose period. All the animals subjected to FOB analysis were handled by single experimenter blindly at the same experimental area. Cage side observations were executed without modulating the existing environmental situation in the experimental set-up.

The following parameters were observed:

Home cage observation	Hand held
Posture	Touch escape
Abnormal vocalization	Reactivity to handling
Tremors	Fur appearance
Convulsions	Salivation Lacrimation Piloerection Exophthalmos Pupil size Body tone Numbness Tingling Itching
Observations in Standard Arena	
Arousal	Hypoactivity
Hyperactivity	Grooming
Palpebral closure	Tremor
Twitches	Clonic convulsions
Tonic convulsions	Ataxia
Hypotonia	Gait
Posture	Stereotypy
Abnormal behaviour	Breathing
Defecation and Urination	Rearing
Manipulative Tests	
Somatosensory/ touch response	Tail pinch response
Visual approach response	Palpebral reflex
Pupillary light reflex	Righting reflex
Auditory startle reflex	

Haematological Estimation: At the end of the experimentation day, isoflurane anaesthesia was administered to the mice in each group. 1 ml syringe was used to aseptically draw cardiac blood. One component of blood was put into an EDTA-containing vial for standard haematological tests. For serum separation, some blood was injected into the clot activator vial.

A semi-automated hemologic analyzer (Horiba ABX Micros ES 60) was used to measure TEC, TLC, and haemoglobin. Fresh blood samples were aseptically taken out and placed on a slide, after preparing a thin smear the slides were stained with Leishman stain. The unusual cytological changes in the WBC, as well as any unique traits were closely scrutinized if any.

Serum enzyme analysis: Blood drawn from the animals for serum analysis was allowed to clot at room temperature for 45 minutes. Following that, the serum was separated in order to measure various biochemical characteristics by centrifuging it at 1000 g for 15 min at 30 °C. The activities ofSerum glutamate pyruvate transaminase (SGPT), Serum glutamate oxalate transaminase (SGOT), alkaline phosphatase (ALP), and

blood urea nitrogen (BUN) were measured using a semi-automated clinical chemistry analyzer (Kaplan et al., 1983).

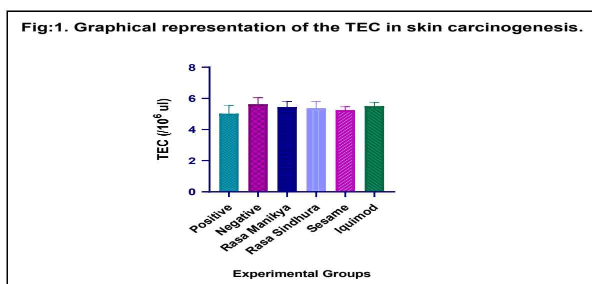
Results and Discussion:

Results of FOB were summarized in the Table 1. All the animals subjected to FOB had been found to be normal in observation time point. Treatment group showed no significant difference in relation to positive group and negative control group.

Haematological parameters provide an accessible way to look for abnormality related to blood having infection. The diagnosis and treatment of a broad spectrum of benign and malignant illnesses affecting the coagulation system, red and white blood cells, and both in adults and children falls within the category of haematology.

The blood was taken at the end of the experiment day and the results were analyzed. Although there was a drop in the Total Erythrocyte Count (TEC) in the positive control group, the TEC count was not significantly lower in the treatment groups. Statistical analysis revealed that there was no significant variance (P≤0.05) among the treatment groups. In comparison to the positive control group, no discernible variation was found between the treatment groups (Figure 1). The most vital component of blood was the red blood cells, which supply the body with nutrients and oxygen. Many hormonal variables contribute in the generation of red blood cells (RBCs) in the bone marrow (Moras et al., 2017). VEGF was essential for the growth and spread of tumours since elevated VEGF levels and receptor activation are linked to a poorer prognosis in cancer patients (Fan et al., 2005).

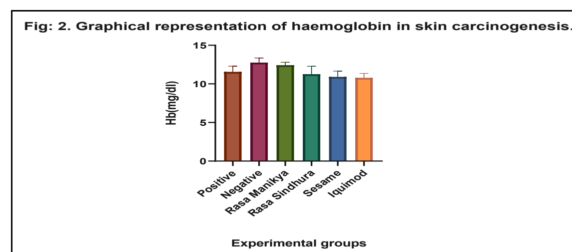
Fig:1. Graphical representation of the TEC in skin carcinogenesis.



Haemoglobin determination was important in assessing the degree and severity of blood loss that occurred during the disease course. Negative group showed an overall constant haemoglobin level. A decline level of haemoglobin was observed in positive control group. Treatment groups *i.e.* rasa manikya, rasa sindhura and sesame oil showed no significant deviation in comparison to positive group (Figure 2).

Statistical analysis: Statistical analysis was done by one way analysis of variance followed by multiple range t test using graph pad prism version 8.

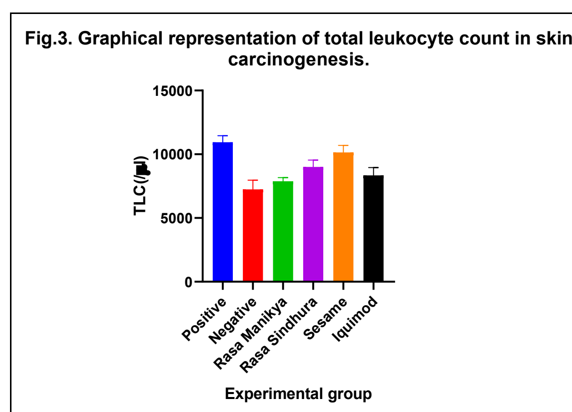
Fig: 2. Graphical representation of haemoglobin in skin carcinogenesis.



Haemoglobin levels are necessary for the transfer of oxygen from carbon dioxide to the tissues. The non-significant decline in haemoglobin count was also linked to factors that lower the RBC count. Hepcidin over expression resulted in the release of various cytokines, such as TNF-α, IL-6, IL-1, and IFN-γ, which stopped haemoglobin from migrating from macrophages into the plasma produced by scenecent RBC recycling (Ganz and Nemeth, 2915). So haemoglobin synthesis was significantly reduced.

White blood cells were crucial in the fight against infection. In this experiment, a noteworthy increase in value was noticed in the positive control group in comparison to the negative group. The increase of leukocytes was non-significantly altered in the therapy group (Rasa manikya, rasa sindhura and sesame) (Figure 3).

Fig.3. Graphical representation of total leukocyte count in skin carcinogenesis.



The first line of protection for cells against infection was WBCs. Polymorphonuclear cell infiltration was frequently observed in cases of inflammation-mediated cancer development. Thus, WBC invasion happened all the time. Consequently, during the course of the experiment, the positive control group's total count grew. (Lee et al., 2006).

Serum enzyme was measured at the end of the experiment and it revealed an elevated level in the animals of positive control group. Though the value was increased in both the treatment groups but it was less than

positive control group. Statistical analysis showed no significant difference in positive control group with negative control group. No significant differences were also observed in between the treatment groups and positive group.

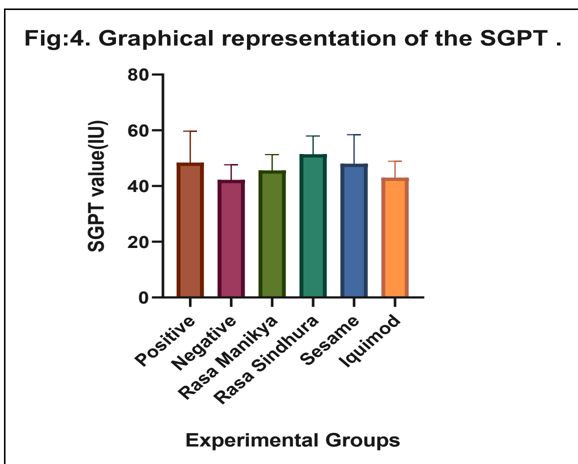


Fig:4. Graphical representation of the SGPT .

SGPT was a significant enzyme that was mostly found in hepatocytes but was also present in almost all other bodily tissues. An increase in this enzyme in the serum could result from any modification to the state of tissue homeostasis. In this study (Figure 4) of two stages carcinogenesis induced the increase production SGPT. Reactive oxygen species were produced as a result of several cytokines and neutrophil infiltration during the disease's development. Hepatocyte injury in the liver was brought on by cytokines and other reactive oxygen species (Li et al., 2015). Rasa manikya showed a non-significant reduction in the value of this enzyme. Ruidas et al., 2019 also demonstrated that rasa manikyais very effective in causing oxidative damage by producing an excessive amount of reactive oxygen species. Reactive oxygenor superoxide can directly interfere with cell metabolism to produce hydroxyl radicals, which in turn can damage DNA, lipid and proteins, thereby can clear pathogen effectively.

Additionally, the positive control group's SGOT value (Figure 5), which was assessed on the final day, was higher. Though not as high as the positive control group, the animals in the treatment group did exhibit greater difference levels of SGOT value. Treatment group's also did not display substantial variation with the positive control group, and a non significant difference was seen between the case positive control group and the negative group.

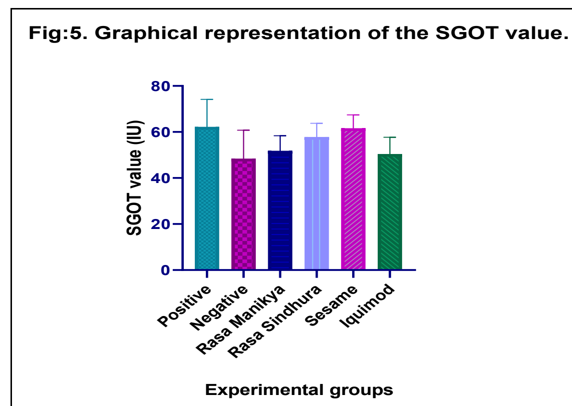


Fig:5. Graphical representation of the SGOT value.

Similar to SGPT, SGOT was a blood enzyme mostly present in the liver and other organs. Thus, an elevated SGOT value may be caused by disruptions in the other organ. Accordingly, the value increased in the positive control group. Both Rasa sindhura and Rasa manikya exhibited hepatoprotective properties although non significantly.

Alkaline phosphatase value measured at the end of the experiment showed rise in the value in all the groups except negative control group. Treatment groups though showed decreased value than positive control group. Statistical analysis demonstrated a significance difference in the positive group compared to negative group. Rasa manikya and iquimod group showed significant change as compared to the positive control group (Figure 6).

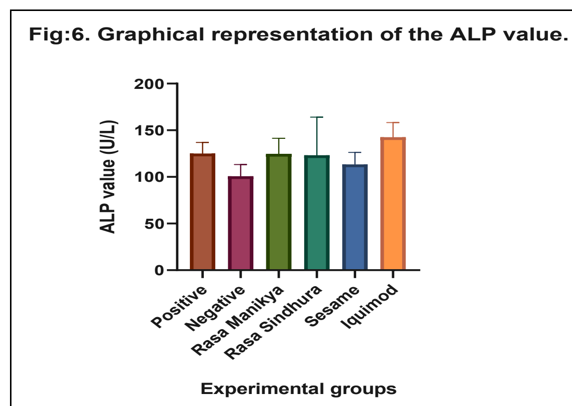


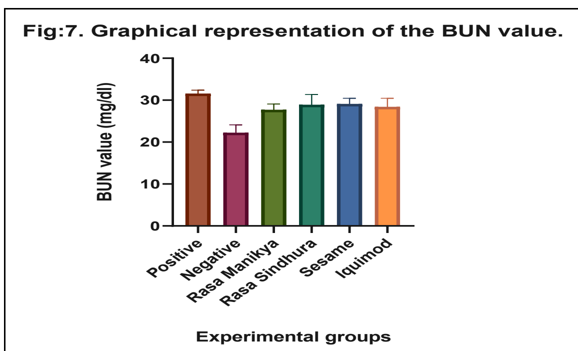
Fig:6. Graphical representation of the ALP value.

ALP phosphatase (ALP) is a class of enzymes that may hydrolyze phosphate esters in an alkaline environment to produce inorganic phosphate and organic radicals. The enzyme was mostly produced by the liver, bones of animals in good health, and in smaller amounts by the kidneys, leukocytes, and intestines. Increased ALP in serum was associated with more cancer-causing infections (Friedman et al., 1996). Hepatoprotective role of rasa manikya and rasa sindhura caused reduction in the ALP level in serum compared to positive control group.

On the terminal day, when compared to the negative control group, the BUN value indicated an increase in the

positive control group. Treatment groups also showed increase BUN value but less in compare with positive control. Non-significant variation observed in the negative and rasa manikya group compare to the positive control group. Rasasindhura and sesame showed no-significant deviation compared to the positive control group (Figure 7).

Fig:7. Graphical representation of the BUN value.



The amount of nitrogen in blood derived from the waste product urea was quantifiable by BUN. The liver produced urea, which the body expelled as urine (Thompson et al., 2020).When an injury-driven inflammatory response transpired, inducible nitric oxide was produced. The body then began to collect urea as a result of this reacting with ROS to form peroxylnitrite and damage renal tubular epithelial cells, which could impair glomerular filtration (Goligorsky et al., 2002). So, positive group showed increased BUN value through the production of an excessive amount of reactive oxygen species. Ruidas et al., 2019 also showed how effective rasa manikya causing oxidative damage. By directly interfering with cell metabolism, reactive oxygen or superoxide can create hydroxyl radicals that can harm proteins, lipids, and DNA, effectively eliminating pathogens.

Conclusion:

Rasa manikya and rasa sindhura both in nanoforms could be used effectively @ 10mg/kg body weight without having much safety concern as shown in this experiment. The enzymes, behavioural pattern and blood pictures shown in this experiment conclusive of chronic therapeutic duration.

Conflict of Interest:

Authors declare no conflict of interest for this study.

Data Availability:

All raw data and backup photography are preserved at the Department of Veterinary Pathology, WBUAFS.

Ethical Statement:

Authors maintained all ethical concern concerns during the experimentation. The study was approved by the Institutional Animal Ethics Committee vide No. ERS/IAEC/2022-2023/INST/004.

Author's Contribution:

MN: Experiment execution and draft manuscript, SM: Experiment Design and manuscript preparation, SB: Planning, resource and statistics, PR: Drug preparation and supply, SP: Haematology, RNH: Behavioural observation and SKM: Enzyme analysis.

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Table **1: Functional Observational Battery (FOB)**

Summary of Functional Observational Battery (FOB)													
Group No., Treatment & Dose (mg/kg/day)		**Positive Control**		**Negative Control**		**Rasa Manikya (10.16 mg/kg)**		**Rasa Sindhura (10.16 mg/kg)**		**Sesame oil (5ml/kg)**		**Iquimod**	
Observation Time Point		**Week 16 Predose**	**Week 16 Postdose**	**Week 16 Predose**	**Week 16 Postdose**	**Week 16 Predose**	**Week 16 Postdose**	**Week 16 Predose**	**Week 16 Postdose**	**Week 16 Predose**	**Week 16 Postdose**	**Week 16 Predose**	**Week 16 Postdose**
Total No. of Mice													
Home cage observations													
Posture	Sitting or standing alert, watching (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
	Sitting normally, feet tucked in (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
	Asleep, lying on side or curled up (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
	Rearing (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Abnormal Vocalization	Absent (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Tremors	Absent (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Convulsions	Absent (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Hand held observations													
Touch Escape	No Resistance (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Reactivity to handling	Squeaks or does not squeak but exhibits mild resistance; easy to handle (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Fur appearance	Normal hair coat	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Salivation	No wetness around mouth (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal

Lacrimation	No Lacrimation (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Piloerection	Piloerection absent (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Exophthalmos	Eye ball centrally located (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Pupil size	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Body tone	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Numbness	Not present	Not present	Not present	Not present	Not present	Not present	Not present	Not present	Not present	Not present	Not present	Not present	Not present
Tingling	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Itching	Not present	Not present	Not present	Not present	Not present	Not present	Not present	Not present	Not present	Not present	Not present	Not present	Not present
Observations in standard arena													
Arousal	Bright, alert and appropriately responsive to the surrounding environment – keeps guard up and engages in exploratory activity (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Hypoactivity	Hypoactivity absent	absent	absent	absent	absent	absent	absent	absent	absent	absent	absent	absent	absent
Hyperactivity	Hyperactivity absent	absent	absent	absent	absent	absent	absent	absent	absent	absent	absent	absent	absent
Grooming	Absent / Present	Present	Present	Present	Present	Present	Present	Present	Present	Present	Present	Present	Present
Palpebral closure	Eyelids wide open (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Tremors	Tremor absent (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Twitches	Absent (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Clonic convulsions	Absent (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Tonic convulsions	Absent (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Ataxia	Ataxia absent (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Hypotonia	Hypotonia absent (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Gait	Head is	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal

	horizontal; abdomen rises slightly above floor, limbs moves in a coordinated manner with slight up and down movement of the body during walking (Normal)												
Posture	Animal walks upright, with the back straight and pelvis off the floor (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Stereotypy	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Abnormal Behaviour	Abnormal behaviour absent (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Breathing	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Defecation	Absent/Present with normal quantity and appearance (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Urination	Absent/Present with normal quantity and appearance (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Manipulative tests													
Somatosensory/ Touchresponse	Locomotor orientation/flinch or startle as an evidence of perception (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Visual approach response	Slowly approaches, sniffs and pulls back/flinch or	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal

	startle as evidence of perception (Normal)												
Pupillary light reflex	Pupil contracts (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Auditory startle reflex	Mild reaction with twitching of the ears or head indicating that the noise was perceived (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Palpebral reflex	Eyelids blinks (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Tail pinch response	Looks back, moves forward and lightly squeaks (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Rightingreflex	Present (Normal)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal

Original Research Article
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Identification of Transposable Elements Responsible For AMR in Food Borne Pathogen

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Abstract

Despite the fact that the presence of silent genes, Transposable elements (TEs) and antibiotic resistance are related, only a small number of studies have examined the presence and distribution of transposable elements in animal food production chains, including pig and poultry farms. It is well known that there is a correlation between the spread of TEs carrying harmful bacteria from farms to food animals and from food animals into the cycle of production of animals for food. The present study identified the presence of five TEs namely Tn6763 (Accession number: OQ565300), Tn6764, (Accession number: OQ565299), Tn6765 (Accession number: OQ409902), Tn2003 (Accession number: OQ503494) and Tn6020 (Accession number: OQ503493) in (N= 235) samples collected from piggery farm and chicken meat shops from Kolkata and some regions of Assam. The identified food borne pathogens mainly belongs to *Enterobacteriaceae* family. Four highly zoonotic food borne pathogens with AMR was identified, 42% (77) *Salmonella enterica*, 26% (49) *E. coli*, 14% (26) *Proteus mirabilis*, 12% (22) *Klebsiella pneumoniae* and 6% (11) *K. pneumonia* were positive and carrying Tn6765, Tn6764, Tn6763, Tn6020 and Tn2003 respectively. Given the presence of TEs responsible for AMR in food borne pathogen and their relative contamination from pig farms and poultry farm to meat shops was described to be a major public health threat. Thus, preventative measures are vital for avoiding the spread of mobile genetic resistance determinants in the Livestock and Animal food production sector and to monitor their emergence.

Key words: Transposable elements, AMR, Food borne pathogens

Introduction:

The mobilization of TEs can pose a risk to the integrity of the genome as they have the potential to cause silent mutations, non-allelic homologous recombination (NAHR), alternative splicing, and various epigenetic changes. This can lead to genomic instability which in turn can accelerate the development of various diseases such as genetic disorders, psychiatric disorders, and cancer (Mukherjee et al., 2004). Antimicrobial-resistant diseases have a huge global health impact. The World Health Organization projects that if no action is taken, there will be 10 million fatalities per year due to drug-resistant bacterial infection by 2050, stressing the crucial need of understanding multidrug-resistant (MDR) microorganisms (World Health Organization, 2019).

Around the world, antibiotic-resistant bacteria are a major contributor to infections connected to healthcare, and resistance has also been observed in infections in the general population. Morbidity, mortality, and medical expenses are all significantly raised by infections brought on by multi resistant organisms. Molecular analyses have revealed that widespread multi resistance has commonly been achieved by the acquisition of pre-ex determinants followed by amplification in response to selection. Mobile genetic elements (MGE), also known as elements that encourage intracellular DNA mobility(for example, from the chromosome to a plasmid or between plasmids), as well as those that enable intercellular DNA mobility, play a significant role in the capture, accumulation, and dissemination of resistance genes (Partridge et al., 2018).

In the present study we collected the different samples from the Pig slaughter houses, pork meat shops, poultry farms and chicken meat shops. Then enriched the samples and further cultured and characterization of bacteria was done. The current study only targeted the gram-negative bacteria, some members of the family Enterobacteriaceae carrying TEs.

Materials and Methods:

Study design, Study area and sampling: The present study was carried from November 2022 to May 2023. Study area was Kolkata metropolitan area and random type of sampling was done (Figure 1).

Sample collection: A total two hundred thirty five (N=235) samples were collected from different locations in Kolkata metropolitan area. One hundred eighty five (185) samples from pig farms and slaughter houses containing feces, soil samples, slaughter effluents,

drainage water, pork carcass/meat washing water, drinking water of animals and pork meat samples. Fifty samples from Chicken meat shops and poultry farms containing Drainage water, feces, soil samples and chicken carcass/meat washing water and chicken meat. All the samples were collected aseptically, samples containing fluids (such as washing water, drainage water etc.) were collected in closed sterile plastic vials and solid (such as feces, soil and meat samples) were collected in sterile polyethylene bags. The samples were collected using sterile cotton swab sticks (HiMedia, India) in sterile vials containing transport medium (HiMedia, India) in accordance with the normal procedure (OIE 2002).The samples were brought to the laboratory in ice pack maintaining proper cold chain. They were held at 4°C for not more than 48 hr before processing.

Enrichment of the samples and culture: The collected samples were enriched in the MacConkey media (broth), (HiMedia, India) and incubated at 37°C for 18-24 hours. Next day growth has been observed in the broth. The enriched samples in MacConkey were sub cultured in the different types of medias such as MacConkey agar, Brilliant Green Agar (BGA), Eosin Methylene Blue Agar (EMB) and *Klebsiella* Blue Agar (KBA) to obtain the pure culture to grow and count microbial cells and to cultivate and select microorganism for further identification.

Gram's staining was used for the initial identification of all the pure isolate cultures in nutrient broth. Gram staining was carried out according to protocol using a smeared slide. Gram positivity or negativity, shape and size of the organisms were taken into consideration. The current study is only targeted towards gram negative bacteria containing Tes responsible for AMR in food borne pathogens

Biochemical assay of the bacterial culture: Various types of biochemical tests such as Indole test, Methyl-Red test, Voges-Proskauer test, Citrate utilization test, Catalase test, Oxidase test and urease tests were done to further characterization of the bacterial isolates.

Primer designing: Primers were designed for cataloguing of different transposable elements existing in the isolated bacterial DNA. Insertion sequences of *Salmonella*, *E. coli*, *Proteus*, *Klebsiella*, *Proteus* of *Enterobacteriaceae* family was retrieved from Isfinder database (Isfinder, https://www-is.biotoul.fr/). Table 1 contains information on the oligonucleotide primers and PCR conditions that were utilized for the identification of transposable elements from *Enterobacteriaceae* family obtained from various sources of piggery waste. The details of the PCR conditions and primers used are provided in the table.

DNA extraction and PCR: The genomic DNA was isolated from an overnight growth bacterial culture using Chen and Kuo's (1993) method with minor modifications. At the end of the exponential phase of growth, bacterial cells were extracted by centrifugation for 5 minutes at 12,000 rpm. The cell pellets were resuspended in lysis buffer (40 mMTris-acetate pH 7.8, 20 mM sodium-acetate, 1 mM EDTA, and 1% SDS), vigorously shaken, and centrifuged for 10 minutes at 4°C at 12,000 rpm. The supernatant was transferred to a new vial, which was then filled with an equal volume of chloroform and inverted many times until the solution appeared milky. The mixture was then centrifuged at 12,000 rpm for 3 minutes, and the supernatant was transferred to another tube for DNA precipitation with 100% ethanol, washed twice with 70% ethanol, air-dried, and resuspended in 1 x TE buffer. Spectrophotometric readings were used to assess the quality and amount of the isolated genomic DNA. Extracted genomic DNA was treated to polymerase chain reaction (PCR) with primers specific for distinct transposable elements. In a 25 µl reaction volume, 10 x PCR buffer (50 mMKCl, 10 mM Tris-HCl, 0.1% Triton X), 5 µlTaq DNA Polymerases, 1 mM $MgCl_2$, 1.5 µM each primer, and 200 µM each dNTP was used. PCR was done for 40 cycles of amplification, with denaturation at 95 °C for 5 minutes, annealing at 55-57 °C for 15 seconds, 72 °C for 15 seconds, and extension at 72 °C for 10 minutes. The PCR results were validated using agarose gel electrophoresis.

Sanger's sequencing and gene bank submission of positive samples: The amplified products of different transposable elements were subjected for Sanger's sequencing for tracing the microbial identity.

Antibiotic susceptibility test: Bacterialisolates encoding different transposable elements were tested for sensitivity/resistance to various common antibiotics such as Chloramphenicol, Amikacin, Gentamycin, Vancomycin, Ofloxacin, Tetracycline, Trimethoprim and sulfamethoxazole., Ampicillin, Nalidixic acid, Cefpodoxime and Colistin (CLSI, 2014).

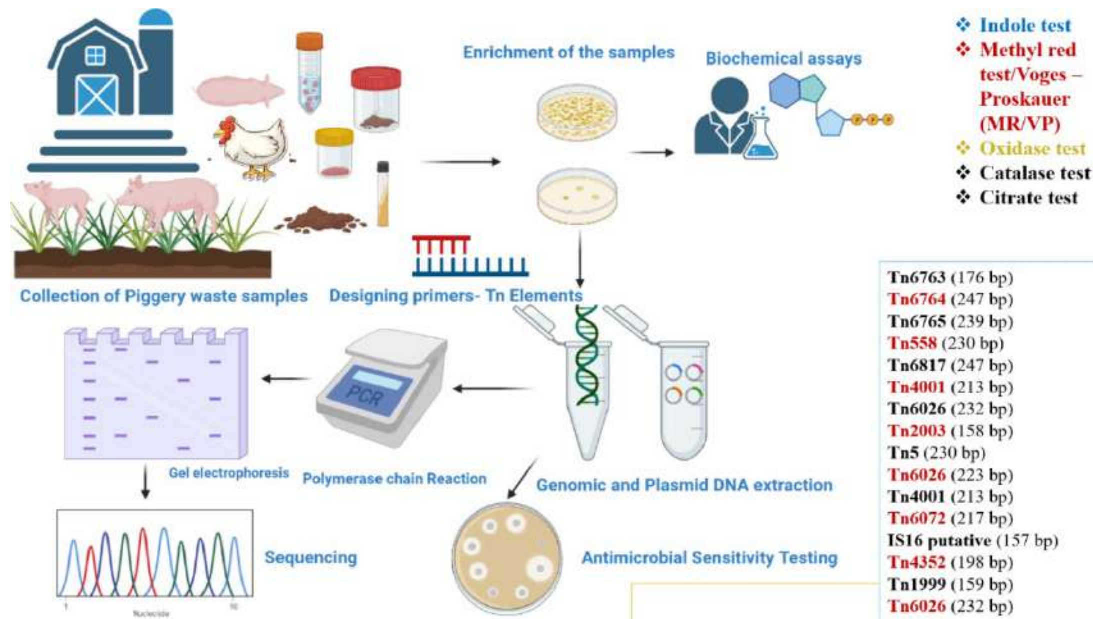

Figure 1: Diagrammatic representation of the methodology used in the study

Table 1: List of the primers used for the current study

TE Name	Forward primer	Reverse primer
1. ***IS16*** **gene**	ACACTGAGTGGCTGGCTTCT	GCCATGCCTTTCTTTCTGAG
2. ***Tn 558***	CTACCCGACATTCCACGACT	ACGGAGTGTGGAGGTTGTTC
3. ***Tn 4001***	TAGCGCGTGAGGCTAAAAAT	GATTGGGGAAGATGCGAATA
4. ***Tn6026***	CACCGGCAGGTGAAGTATCT	CAATGCCAAAAGCTCTCTCC
5. ***Tn 6020***	CAACGTGAAGAAGTGGCAGA	ACCTTTGATGGTGGCGTAAG
6. ***Tn2003***	GAGCTGCACATGAACCCATT	CAGCGGTAAATCGTGGAGTG
7. ***Tn5***	CGGATCGAGGAGTTCCATAA	TTCTACGTGTTCCGCTTCCT
8. ***Tn2006***	GAGTCACTGGCGTGTTCTCA	CGTCCGGAAAAGTTCATTGT
9. ***Tn6072***	AACGCGGTTTACAAGGTACG	GCCTCACGCGCTAAGTTAAT
10. ***Tn5384***	AGATCTGAAGAGACCTGCGG	AGATCAGGCACCTTCAACGA
11. ***Tn1999***	CAACAGTCCAGAGCGATTCG	CCCAAGCGAATCGTTGAGAG
12. ***IS Sav10***	GCTTCCTCTGCCATTCGATG	CCGATGTTCTGCGACGAATT
13. ***Tn 6763***	GTTGGTGCACAAAACATTCG	TGCTTTTCAGAAGGCAAGGT
14.***Tn 6764***	TTCAGGCACGTATGTGGGTA	GCTTGAGGCGTTTATTCAGC
15. ***Tn 6765***	ACCGACAGCGATACGGTAAG	ACGGTGTGTATGTCGAGCAA
16. ***Tn 6718***	GCTTGAGGCGTTTATTCAGC	TTCAGGCACGTATGTGGGTA

Results and Discussion:

Isolation of Enterobacterials: To isolate *Enterobacteriaceae* bacteria, we used various agar media MacConkey, EMB, BGA, and *Klebsiella* selective agar. Out of the total samples collected, 185 (78.72%) were found to have grown on the fore mentioned media and were selected for primary identification through Gram staining (Figure 2).

DNA extraction and PCR based detection of TEs: Out of the total number of samples (N=235), 185 were found to be positive for Enterobacterial family. To identify any transposable elements, present in these samples, we screened each sample using 16 primers that were designed specifically for this purpose. As a result of this screening, we obtained positive with five transposable elements (Figure 3).

Transposable elements identified in samples and their percentage positivity: Out of 185 samples, 42% (77) *Salmonella enterica*, 26% (49) *E. coli*, 14% (26) *Proteus mirabilis*, 12% (22) *Klebsiella pneumoniae* and 6% (11) *K. pneumonia* were positive and carrying Tn6765, Tn6764, Tn6763, Tn6020 and Tn2003, respectively (Figure 4).

Sanger sequence analysis and gene bank submission: The sequencing results were analyzed Figure 5 and Figure 6, and the sequence was submitted to www.ncbi.nlm.nih.gov to obtain an accession number. Results of sequencing and accession no. obtained. The present study identified the presence of five TEs namely Tn6763 (Accession number: OQ409902), Tn6764, (Accession number: OQ565299), Tn6765 (Accession number: OQ409902), Tn2003 (Accession number: OQ503493) and Tn6020 (Accession number: OQ503493)

Antibiotic resistance or susceptibility pattern and determination of minimum inhibitory concentration of bacteria: All the bacteria which were identified by the sequencing & were carrying transposable elements positive were screened with antibiotics. *Klebsiella* spp. Carrying Tn6020 and Tn2003 isolates were resistant (100%) to Nalidixic acid, vancomycin, ceftriaxone, ceftazidime, cefotaxime, cefpodoxime, ceftizoxime, cefixime, amoxicillin-clavulanate combination, tetracycline, gentamicin, ciprofloxacin, levofloxacin, amikacin, doxycycline and cefepime/tazobactam. Again, *Proteus mirabilis* is Carrying Tn6763 (Figure 7) were resistant to piperacillin/tazobactam, and cefepime, chloramphenicol, streptomycin and trimethoprim/sulfamethoxazole, For Tn6764 &Tn 6765 which was carrying bacteria *E. coli* and *Salmonella enterica* were resistance Ofloxacin (Figure 7) frequently noted to other antibiotics like norfloxacin (85%), tobramycin (65%), cotrimoxazole (60%) and cefoxitin (60%). It is noteworthy that almost all the bacteria isolates were intermediate resistant to imipenem. Most of the isolates were sensitive to Chloramphenicol (60%), Amikacin (100%), meropenem (100%) and colistin (100%).

To spread bacterial AMR, Tn7-like transposons are crucial mobile elements. The information currently available on the thorough analysis of Tn7-like transposons in Enterobacterales isolates is still insufficient when compared to studies about the transposition mechanism of the Tn7-like transposons. In this study, we evaluated the percentage positivity of Tn7-like transposons in isolates of the genus Enterobacterales isolated from various farms of pigs raised for meat. Among the 235 sample 180 were positive for Enterobacterales isolates. The positive rate of Tn7-like transposons in *Proteus* spp. (50.6%) was significantly higher than other bacteria. This finding was consistent with previous reports that genomes of *Proteus* spp. exhibited strong plasticity facilitating high-frequency insertion of mobile genetic elements like Tn7-like transposons. (Dong et al., 2019, Gu et al., 2020).

The characterization of individual TEs and the recombination events they predict has been the subject of numerous studies. Positive antibiotic selection can easily identify such AR-causing events. However, focused analyses of global omics are required to fully understand the effect of TE-dependent changes on bacterial transcriptomes. Understanding the molecular underpinnings of the operation of a much larger pool of TEs, representing various families of elements, is equally crucial. The discovery of new pathways and mechanisms that activate silent genes may be made possible by a better understanding of their biology and unique properties.

Significant increases in the emergence and spread of bacteria that are resistant to multiple drugs as well as rising resistance to more recent drugs like fluoroquinolones and specific cephalosporins have been observed over the past two decades (von Baum et al., 2005). The development of antimicrobial resistance in *E. coli* has been attributed to a variety of mechanisms, with horizontal gene transfer through conjugative plasmids, transducing phages, and transposable elements carrying resistance genes making the biggest contributions to this genomic fitness.

Our findings show that there may be a reservoir of Tn7-like transposons in the Enterobacterales strains, which is a risk that requires our attention. Antimicrobial susceptibility testing indicated that Enterobacterales strains carrying Tn7-like transposons exhibited high resistance to a variety of antibiotics, with a 54.9% multidrug resistance rate. Relatively high rates of MDR in *Proteus* spp. and *S. enterica* strains may be due to the prevalence of multiple mobile elements in both bacteria, which was confirmed in previous studies (Beutlich et al., 2011; Murgia et al., 2015; Schultz et al., 2015; Lei et al., 2018)

Additionally, it suggested that Tn7-like transposons were probably to appear concurrently with other mobile elements. The intI2-associated resistance gene cassette (aadA1, sat2, and dfrA1) carried by Tn7-like transposons is responsible for the high resistance to streptomycin (87.8%) and trimethoprim/sulfamethoxazole (74.0%,), (Tietze and Brevet et al.,1991; Kaushik et al., 2019).The gene cassettes of intI2 contained the aminoglycoside adenyltransferase (aadA1 and aadA2), dihydrofolatereductase (dfrA1), and streptothricinacetyltransferase (sat2) encoding genes, which are responsible for streptomycin-spectinomycin, trimethoprim, and streptothricin resistance, respectively (Zhang et al., 2019)

Compared with the resistance rates to streptomycin and trimethoprim/sulfamethoxazole, relatively low resistance rates to florfenicol (60.5%), gentamicin (24.4%), and ciprofloxacin (22%) were detected in the Tn7-like transposons positive strains. The majority of them

demonstrated susceptibility to the antibiotics imipenem, ceftazidime, cefoxitin, aztreonam, and amikacin. In China the abuse of antibiotics as feed additives in veterinary clinical practise and the transfer of movable components carrying drug-resistant genes between isolates, which has become a common problem in both human and veterinary clinical practise, are the main causes of the emergence of severe drug-resistant status (Rahmani et al., 2013).

Figure 2: Isolation of the members of the family Enterobacteriaceae showing different bacterial strains such as *E. coli, Salmonella, Klebsiella* in differential and selective media

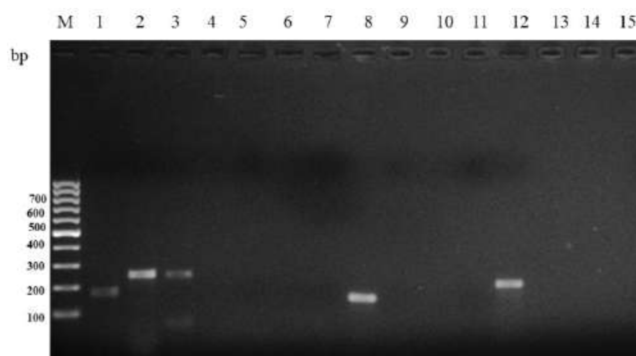

Figure 3: Amplification and detection of 5 Transposable elements; Lane 1: Tn6763(176bp); Lane 2: Tn6764 (247bp); Lane 3: Tn6765 (239bp); Lane 8: Tn 2003 (158bp) and Lane 12: Tn6020 (232bp) in enterobacterial isolate, (*Proteus mirabilis, Escherichia coli, Salmonella enterica, Klebsiella pneumoniae, K. pneumoniae*). Lane M: molecular mass marker (100 bp, ThermoFisher scientific) representing 176bp, 247bp, 239bp, 158bp and 232bp.

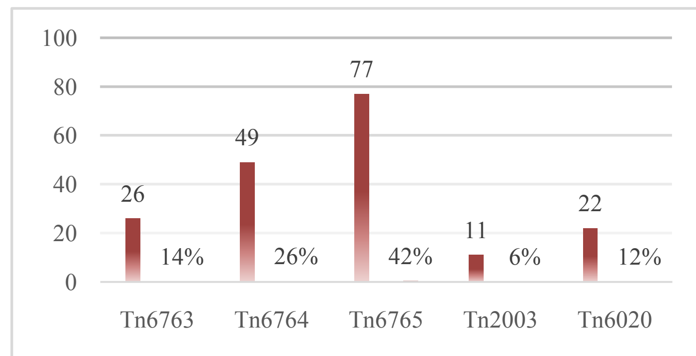

Figure 4: Percentage positivity with TEs; 42% (77) *Salmonella enterica*, 26% (49) *E. coli*, 14% (26) *Proteus mirabilis*, 12% (22) *Klebsiella pneumoniae* and 6% (11) *K. pneumoniae*were positive and carrying Tn6765, Tn6764, Tn6763, Tn6020 and Tn2003 respectively

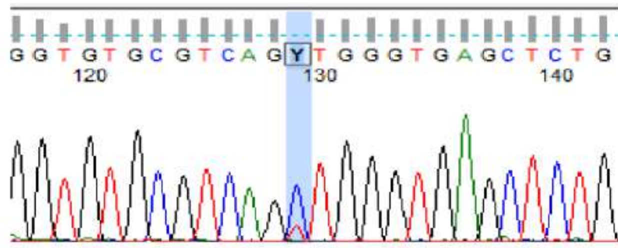

Figure 5: Direct sequencing of Tn6765 amplified Fragment Showing heterozygous locus at position 159.C > T

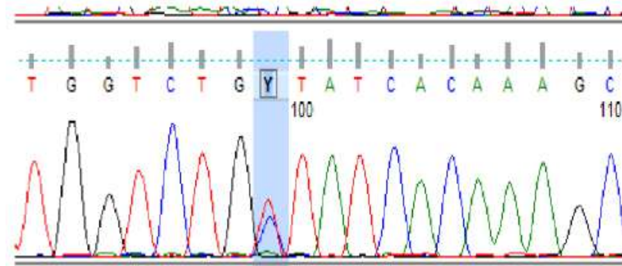

Figure 6: Direct sequencing of Tn6763 amplified Fragment Showing heterozygous locus at position 129.T > C

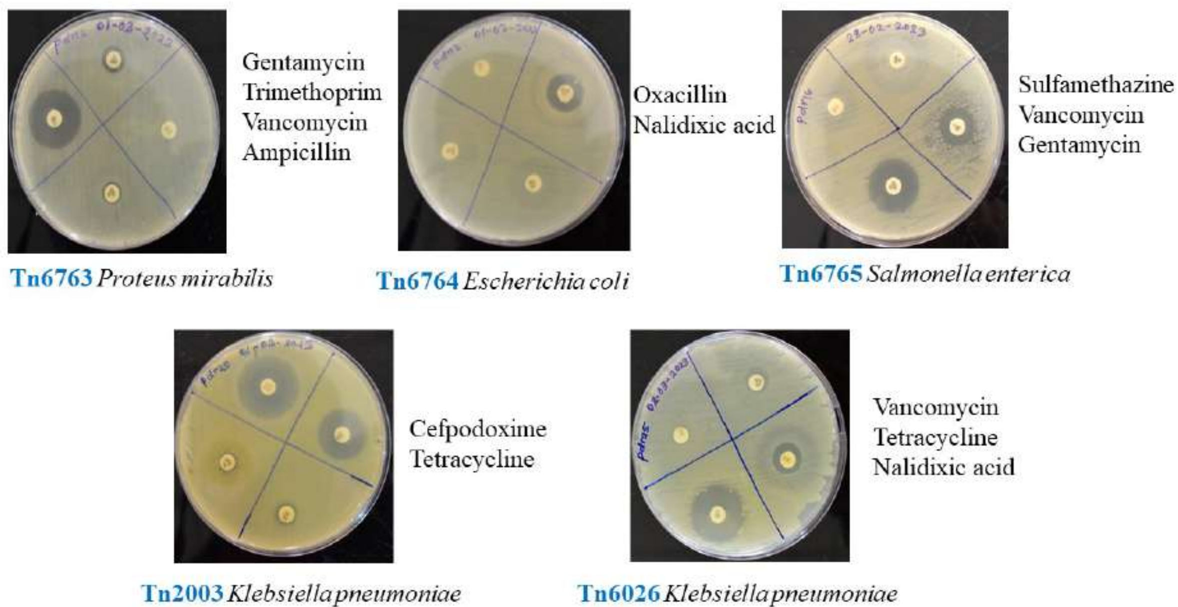

Figure 7: Antibiotic sensitivity tests of the positive samples (Tn6763 *Proteus mirabilis*resistant to Gentamycin, Trimethoprim and Ampicillin), (Tn6764 *E. coli* resistant to Oxacillin and Nalidixic acid), (Tn6765 *Salmonella enteric* resistant to Sulfamethazine, Vancomycin and Gentamycin), (Tn2003 *K. pneumonia* resistant to Cefpodoxime and Tetracycline) and (Tn6026 *K. pneumonia* resistant to Vancomycin, Tetracycline and Nalidixic acid)

Conclusion:

High rates of MDR in *Proteus* spp. and *S. enterica* strains was due to the prevalence of multiple mobile elements in the bacteria. Enterobacterales strains carrying Tn7-like transposons exhibited high resistance to the antibiotics. This study concluded that it is very crucial to standardize clinical drug application to increase the efficacy of new medications.

Conflict of interest:

Authors declare no conflict of interest for this investigation.

Data Availability:

All raw data are preserved at the ICAR-National Research Centre on Pig, Guwahati, Assam, India

Ethical Statements:

Authors maintained all ethical concern during sampling and collection of data throughout the experiments.

Author's Contribution:

PKT: Study Execution**, RP:** Sample collection**, SRP:** Sample analysis and supervision, **RD:** Molecular study**, JN:** isolation study**, PJD:** preparation of the manuscript**, GSS:** Isolation **, JS:** sequence analysis**, VKG:** revision of manuscript.

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A Case Report on Canine Transmissible Venereal Tumor

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Abstract

A female Labrador dog 4 years was presented in Teaching Veterinary Clinical Complex, Bihar Veterinary College, Patna, Bihar Animal Sciences University Patna, Bihar with a complaint of swollen masson the ventral side of the neck region. After preparing the Fine Needle Aspiration Cytology (FNAC) of the tumorwas done and observation was made under an oil immersion microscope at 100x magnification. The microscopic examination revealed the presence of cytoplasmic vacuolations, leading to the diagnosis of Canine Transmissible Venereal Tumor (CTVT). Chemotherapy was initiated using the most effective cytostatic agent, Vincristine sulfate, administered intravenously once a week. The chemotherapy regimen was repeated until the tumor completely regressed, and the condition was successfully resolved after the third chemotherapy session.

Key words: TVT, FNAC, Cytology, Vincristine sulfate.

Transmissible Venereal Tumor (TVT) is a tumorous condition transmitted horizontally among dogs after coitus mediated by the viable tumor cells possessing transposons (Spugnini et al., 2007). TVT can also be called as Sticker tumor or sarcoma, transmissible lymphosarcoma, venereal granuloma, infectious granuloma, canine condyloma and contagious lymphosarcoma (Murgia et al., 2006). Novinsky in 1876 initially described canine TVT and demonstrated that the tumor could be transferred from one host to another via tumoral cells (Richardson, 1981). This viable tumor cell mainly affects the genital region whereas extra-genital cases of TVT have also been discovered and treated. Extra-genital region includes nasal cavity, conjunctiva and eye, skin, neck and thigh region (Barron et al., 1963). This might also lead to the condition known as phimosis. TVT cells are round with large round nuclei that possess coarse chromatin and single, prominent nucleoli. It also consists of prominent cytoplasm with distinct vacuolation (Marchal et al., 1997). The tumor growth occurs 15 to 60 days after implantation of tumor cells. Normal canine cells possess 78 chromosomes whereas CTVT cells consist of 57-59 chromosomes (Prier, 1966). Some cases reported spontaneous regression of tumor mass and also the recovered dogs were found to acquire humoral and cellular immunity against CTVT (Beschorner et al., 1979). Metastasis was found to be seen in less than 5-20% of cases and was more common in male (15.8%) than in female dogs (1.8 %) (Rogers, 1997). The rapidity of metastases depends on the health and immune status of the affected dog.

A 4 years old female Labrador weight of 22kg was presented in Teaching Veterinary Clinical Complex, Bihar Veterinary College, Patna, Bihar Animal Sciences University Patna, Bihar with a complaint temperature of 106.5^0 F, Anoxia, and loss of body weight with large swollen mass on ventral side of neck region. On physical examination, the nodular, lobulated mass was seen at the neck region. Grossly, the mass was nodular, raised toneck region (Figure 1).

Figure 1: Tumorous mass in neck region

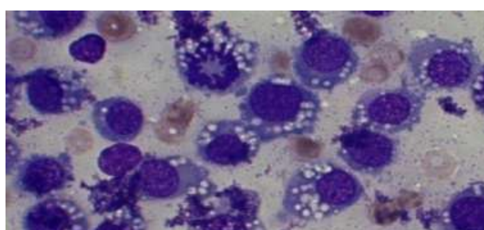

Figure 2: The fine needle aspiration cytology was prepared and shows the presence of cytoplasmic vacuolations after microscopic observation (100x).

The condition was tentatively diagnosed to be CTVT based on the expertise of the clinician. CTVT was mainly diagnosed by impression smear of the tumor or by using fine needle aspiration cytology (FNAC). So, the fine needle aspiration cytology (FNAC) was preceded at Department of Veterinary Pathology, Bihar Veterinary College, Patna, Bihar Animal Sciences University, Patna. The smear was then fixed with alcohol and was stained

using Wright-Giemsa stain. The stained smear was observed using an oil immersion microscope (100x). Under the microscope confirmatory structure was visible, i.e. highly vacuolated cytoplasm with few numbers of mitotic changes in few numbers of cells.

Chemotherapy was found to be the most effective method of treatment among all other. Complete regression of tumor takes 2 to 8 injections in most of the cases, and has a good prognosis with chemo therapy. Among various cytostatic agents, vincristine sulfate was found to have a higher success rate. So, vincristine sulfate (C-VINLONTM @1mg/ml) was administered at the rate of 0.025-0.030 mg/kg body weight intravenously (Cephalic vein) once a week. Vincristine was given with fluid (i.e. normal saline at the rate of 2drops/second) to reduce the burning sensation in veins. Chemotherapy was performed for three sessions and the condition was resolved. The prognosis of CTVT of the extra-genital region was excellent after the treatment with vincristine sulfate.

TVT is mostly benign in nature which was found to be transmitted directly from dog to dog, across major histocompatibility complex (MHC) barriers and also through damaged mucosal surfaces mediated by the viable tumor cells during mating (Igor et al., 2012). TVT possess unique characteristics of occurring naturally and transmitted as an allograft which acted more or less like a parasite that grew autonomously from the original host. CTVT cells could avoid its detection from immunological cells by inhibiting MHC-II activity or down-regulating MHC-I activity due to the secretion of inhibitory cytokines i.e., IL-6 and TGF-1 (Axnér et al., 1996). Treatment of TVT mainly included surgical techniques, radiotherapy and chemotherapy. Surgical method of treatment was being used since last century with lower efficacy. Small, localized TVTs were treated extensively using a surgical method in spite of its higher chances of reoccurrence (Marchal et al., 1997). The use of electro-cautery in surgery proved to have a higher success rate. Vincristine sulfatewas obtained as the salt of an alkaloid from a common flowering herb, the periwinkle plant (Vincarosea Linn). The exact mode of action of vincristine sulfate is still under investigation. But some researches had shown that it inhibited microtubule formation in the mitotic spindle resulting in arrest of mitotic division of cells at metaphase stage. Extravasation of anti-neoplastic agents like vincristine showed symptoms ranging from local pain, inflammation and ulceration vinblastine, vinorelbine (Gilbar and Carrington, 2006).

CTVT was the unique type of tumor of benign nature mostly seen in genital regions. Vincristine was found to be effective even in the case of venereal tumors of the extra-genital region. Animals must be prevented from having intercourse with random dogs to prevent transmission of CTVT. The rate of fluid administration along with chemotherapeutic agent must be maintained at an optimum rate to prevent burning sensation suffered by the drug. Regular administration of chemotherapeutic agents must be done weekly till the resolution of the condition.

Conflict of interest:

Authors declare no conflict of interest for this investigational report

Data availability:

All raw data and backup photography are preserved at the Department of Veterinary Pathology, Bihar Veterinary College, Bihar Animal Sciences University, Patna

Ethical Statement:

Handling of the dog during the treatment was performed under the ethical guidelines of IAEC, Bihar Veterinary College, Patna.

Author's contribution:

DK: Sample collection and manuscript preparation, **RP:** Sample collection and staining, **MAG**: Preparation of draft manuscript, **PKB**: Photography and data collection and **AK:** Cytology and sample preparation.

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