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Original Research Article

A histopathological study of virulence of food borne pathogen *Listeria monocytogenes* NCTC 7973 undergoing animal passage

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Abstract

Pathogencity of Listeria species has been related to the production of cytolysin to induce lesions and to damage the host organs. L. monocytogenes NCTC 7973 was passage through rabbits and severity of infection before and after passage was determined by histopathological studies of the infected organs in the mice In the present study it was found out that virulence of the strain was enhanced several time after successive inoculation and reisolation from natural host (rabit). Experimental listeriosis was produced in a group of experimental mice by inoculating L. monocytogenes NCTC 7973 through intraperitoneal route. When the passage culture of L. monocytogenes was injected intraperitonealy, characteristic lesions of the liver and lymphoid organs including mesenteric and lumber lymph nodes with multiple focal areas of necrosis appeared within 24 to 48 hours. Whereas, lesions in the spleen appeared after 48 to 96 hours. In contrast when the unpassage culture of L. monocytogenes was injected, the lesions in the liver appeared after 72 hours with less necrosis.

Keywords*: Listeria*, Lesions, Pathogenicity and Histopathological

INTRODUCTION

Bergey's Manual of Systematic Bacteriology, 9th edition (26), lists eight species in the genus Listeria: *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. ivanovii*, *L. grayi*, *L. murrayi*, L. *denitrificans*. Both *L. ivanovii* and *L. monocytogenes* are pathogenic for mice, but *only L. monocytogenes* is consistently associated with human illnesss (Cooper and Walker, 1998). *Listeria monocytogenes* was recognized as causing human foodborne illness in 1981.

Intracellular facultative *Listeria monocytogenes* is found in multiple ecological sites, which allows an easy access to food products (Huang et al., 1990; Kuhn and Goebbel, 1989). The WHO instituted a Zero tolerance policy for *L.* monocytogenes in ready-to-eat products because of increasing concern regarding potential for growth of Listeria in processed food (Bryan, 1992). Listeriosis is the acute illness caused by an infection from the bacterium. Listeriosis is most common among elderly persons, neonates, immunocompromised individuals and pregnant women. It may cause sepsis syndrome and premature labour or foetal death during pregnancy (Allerberger and Guggenbichle, 1989; Posfay-Barbe and Wald, 2009; Hamon et al., 2007). *L. monocytogenes* has the ability to cross tight host barriers, including the intestine, blood–brain barrier (Taekyun et al., 2000), and fetoplacental barrier (Hamon et al., 2007).

Listeriosis is one of the seasonal CNS diseases in domestic animals including cattle, goats and sheep (Gandhi and Chikindas, 2007; Lecuit, 2007; Liu et al., 2006). Some adults may also be at risk from more severe complications and death from listeriosis. The incubation period for listeriosis is 4 days to several weeks. Foods that are typically linked to this bacterium are raw milk products, vegetables, seafood, poultry, red meat, liquid

whole egg, and ready-to-eat foods such as hotdogs and luncheon meats (Klara et al., 2004; Brouwer et al., 2006).

Virulence of Listeria has been studied in different ways. It is clearly established that pathogenicity is a multifactor process and has been investigated as a function of the activity of different metabolites, which are necessary for evasion from the cellular defense mechanisms (Chakraborty and Goebel, 1988). These include factors involved in adherence [9], capability to infect macrophages, survival and growth in the host cells, cell-to-cell spread and to the induction of lesions and death in experimentally infected mice (Galsworthy, 1987; Marco et al., 1992).

Over the past years, listeriosis has been a subject of considerable interest and there has been a tremendous advance in our knowledge of listeriosis epidemiology. The genetic lineages of *L*. monocytogenes strains are classified into three lineages I, II, and III, lineage I having the group of serotypes 1/2b, 3b, 3c, and 4b,mainly contributes to the major epidemic clone strains, implicated in multiple listeriosis epidemics in the world. Whereas,lineage II belonging to serotypes 1/2a, 1/2c, and 3a, sequested in clinical cases of human. The lineage III have 4a and 4c serotype usually found in ruminants (Bille and Doyle, 1991; Stelma et al., 1987).

In the present virulence studies, effect of animal passage on the virulence of the bacterium was determined by the histopathological studies.

MATERIALS AND METHODS

Animals

The following experimental animals were used:
Rabbits (local breed) ranging from 1500-1800g in weight.
Mice (strain NMR) weighing 18-20 g and 3-4 weeks of age.
Animals were kept under sanitary conditions in the animal house of the H.E.J Research Institute of chemistry, University of Karachi, fed conventional diet and given water ad libitum.

Bacterial strains and preparation of inocula

L. monocytogenes NCTC 7973 serover ½ was maintained on Tryptone Soya Agar (TSA, Oxoid) slopes at 4°C and their identity was confirmed by microscopy, cultural characteristics and biochemical tests.

Culture was grown overnight at 37°C in 100ml TSB. Cells were harvested by centrifugation (3,500 x g for 30 min) and suspended in sterile phosphate–buffered saline (PBS), pH 7.2,to an optical density at 500nm of 0.5 (approximately 5 x 10^9 CFU ml^{-1}). Cell suspension was serially diluted 1:10 in sterile PBS and surface inoculated onto duplicates plates of TSA and enumerated after 48 h of incubation at 37°C.

Inoculation procedure and reisolation

The culture was serially passage in rabbits successively for six times. For challenging the animals, one ml of standardized suspension ($1x10^9$ cells) of the of *L. monocytogenes* was injected intravenously into healthy rabbits with negative blood cultures. Animals were observed for characteristic symptoms of listeriosis until death.

Liver, spleen and brain of the dead animals were aseptically removed, chopped in sterile PBS and 1.0 gram of each was transferred to 9 ml of TSB; (with potassium tellurite and thallous acetate added to a final concentration of 1%). Suspensions were serially diluted 10^{-1} through 10^{-10} in TSB and 0.1 ml of each dilution was plated on TSA plates. Listerial growth was confirmed by morphological, cultural, biochemical and serological tests and this culture was used to inoculate fresh animals.

Assay for determining the virulence of test strains before and after six-time animal passage was done by making ten fold and two fold dilutions in sterile PBS. A group of six mice was inoculated intraperitoneally(i.p) with 0.5 ml of appropriate dilution. At an interval of 24 hours, selected mice from each group were killed sequentially and gross lesions were observed. Specimen from different organs particularly liver, spleen, lung and heart were cultured in TSB containing 0.02% thallous acetate, homogenized and plated.

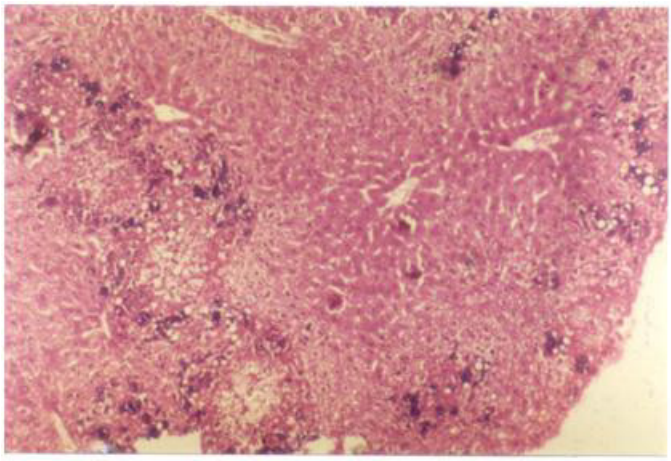
Histopathological examination

Tissue samples from different organs particularly spleen, liver, lung were embedded in paraffin, and 4 micron microscopical sections were cut and stained with haematoxylin and eosin (H&E) stain.

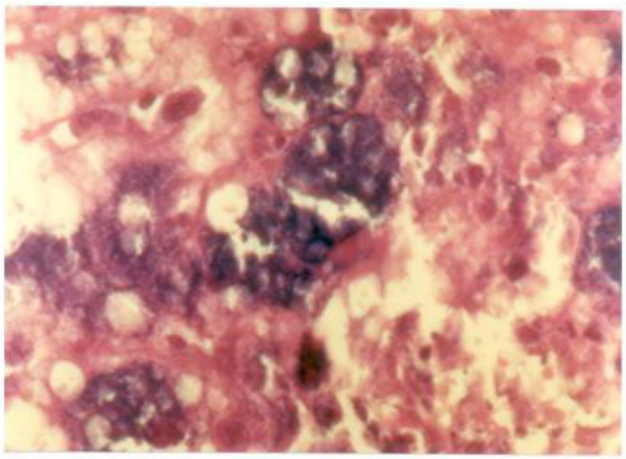
RESULTS

Clinical picture

Mice injected with *L. monocytogenes* cells (passaged/ unpassaged) showed symptoms of illness after 24 hours. The first apparent symptoms were the appearance of pilorection, hypothermia, slight increase in respiration and ptosis. During the following 24 hours the eyes were filled with thick muco-purulent discharge.The mice appeared emaciated indicating loss of appetite and mucoid diarrhoea. In case of passage culture, those mice injected with large doses seemed to die of the overwhelming infection within 20 to 24 hours after the appearance of the first symptom. In some however, after



Photomicrograph 1a. Section of mice liver inoculated with ***L*** monocytogenes NCTC 7973 (passaged). Picture showing large areas of multiple microabcesses with loss of liver cell details. Aggregates of *L* monocytogenes stained deeply are seen (low power, H & E stain).

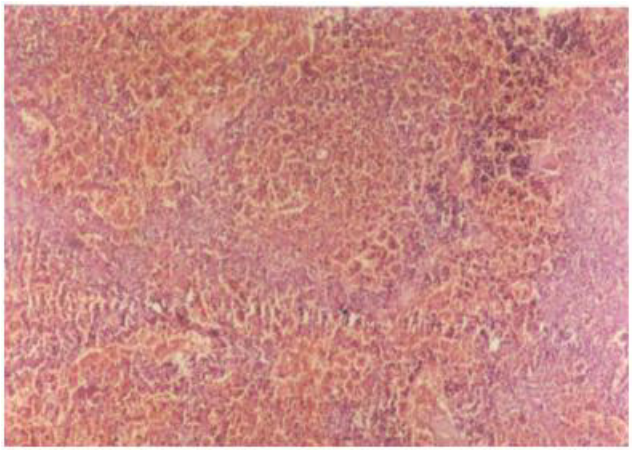


Photomicrograph 1b. Section of mice liver inoculated with L monocytogenes NCTC 7973 (passaged). Picture showing complete loss of lobular/cord like structure of liver cell and dense Aggregates of *L* monocytogenes stained deeply (high power, H & E stain).

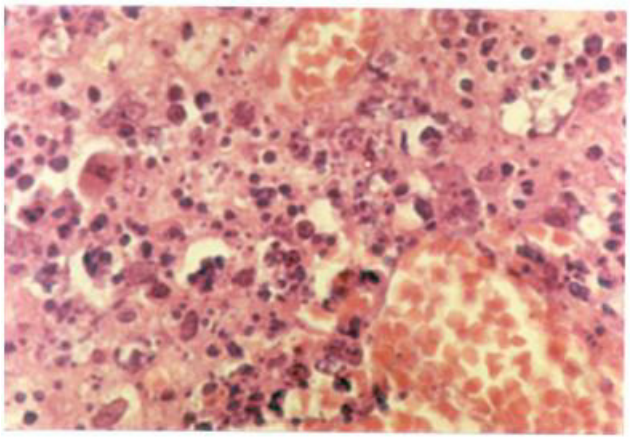
5 days there was a partial paralysis of the hind limbs followed by death.

Gross Findings

In case of *L. monocytogenes* NCTC 7973 (unpassaged) many of the mice, which were dead within 48-72 post-inoculation hour (PIH) did not show evidence of necrosis or other visible pathological changes either disease showed in liver, spleen, or lungs. In contrast, mice which underwent a long course of the disease showed characteristic gross lesions in the liver and lymphoid organs, including spleen, maxillary, mesenteric and lumber nymph nodes with multiple pale to gray foci (1-2 mm in diameter). Development of liver cell necrosis showed a mottled appearance to the organ. Followed by an increase in the number and size of the necrotic areas of the hepatic foci (4-6 mm in diameter). Prominent mixed inflammatory infiltration or narcotizing lesions were observed in the spleen. However, mice that were inoculated with the same strain after being passaged (6 passages) showed consistently extensive damage and necrosis of the liver and spleen. Even they were dead within 12-24 PIH. Some of the dead mice showed evidence of spleenomegaly. In case of *L. monocytogenes*



Photomicrograph 2a. Section of mice spleen inoculated with L monocytogenes NCTC 7973 (passage). Picture showing red pulp and white pulp area, numerous areas of necrosis loss of normal spleenic architecture (low power, H & E stain).



Photomicrograph 2b. Section of mice spleen inoculated with L monocytogenes NCTC
7973 (passaged). Picture showing numerous inflammatory cells, red cells and destruction of spleen structure. (high power, H & E stain).

unpassaged culture the number and size of the necrotic foci was less as compared to that of passaged culture. Lung, and heart of such cases did not show visible changes.

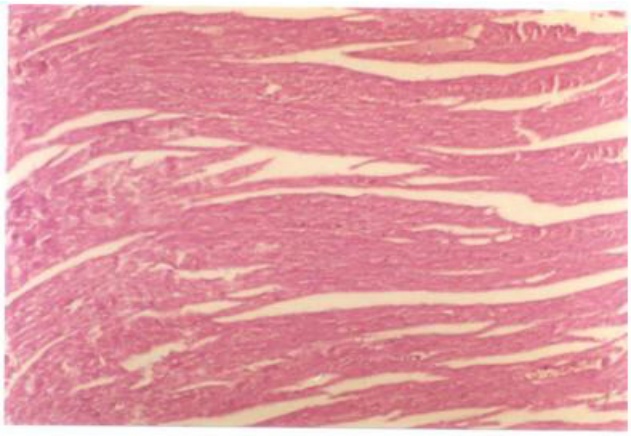
Microscopic Findings

In case of mice infected with *L. monocytogenes* NCTC 7973 (passaged) culture. Distribution of the lesions in the liver and spleen were consistent with an infection as seen in severe bacteremia, which revealed the development of a cellular inflammatory response. Numerous necrotic foci were observed in a single lobule, sometime without affecting the adjacent lobule. The necrotic foci consisted of an area of necrosis with fragments of hepatic cells and necrobiotic polymorphnuclear cells as shown in Photomicrograph 1a.

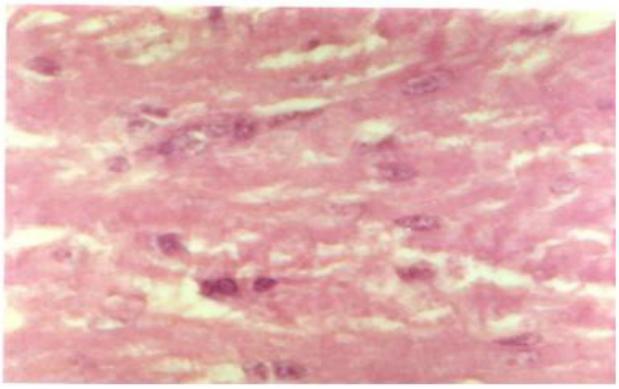
Furthermore, there was an enlargement of the individual focus and confluence of adjacent foci, resulting in larger areas of necrosis containing degenerating leukocytes and cellular debris from hepatocytes, complete loss of lobular, cord like structure of liver (Photomicrograph 1b).

Similar patterns of infection follows in the spleen revealed multifocal necrosis and an increased number of neutrophils and macrophages (Photomicrograph 2a, 2b). The infected heart of the same animal shows thick and inflamed striated muscles, with areas of mild necrosis, indicating the inflammatory response (Photomicrograph 3a, 3b).

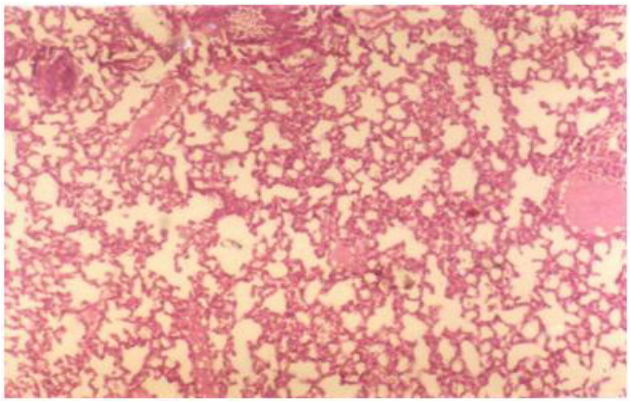
The long also showed focci of necrosis, but intensity is



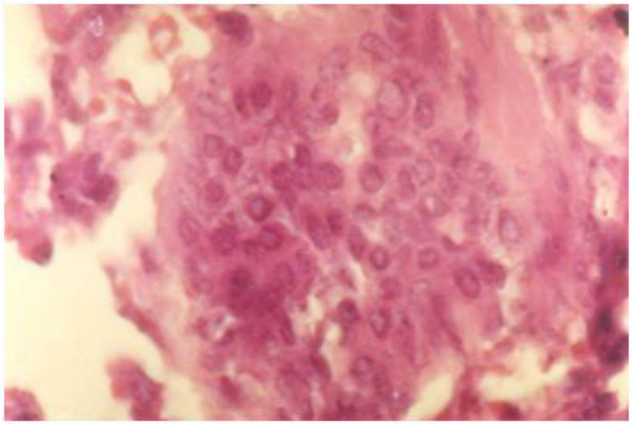
Photomicrograph 3a. Section of mice heart inoculated with L monocytogenes NCTC
7973 (passaged). Picture thickened, inflamed striated muscles of heart indicating inflammatory response (low power, H & E stain).



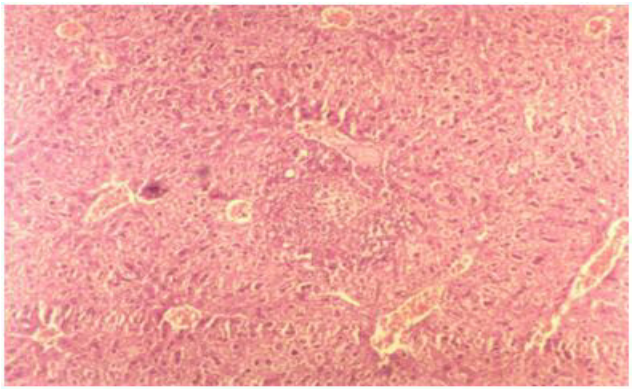
Photomicrograph 3b. Section of mice heart inoculated with L monocytogenes NCTC
7973 (passaged). Picture showing loss of normal structure of heart musculature with area of mild necrosis and collection of inflammatory (high power, H & E stain).



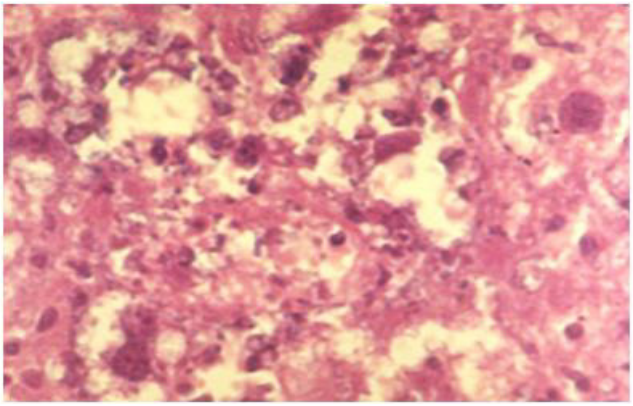
Photomicrograph 4a. Section of mice lung inoculated with L monocytogenes NCTC
7973 (passaged). Picture showing foci of necrosis, along with numerpus alveoli seen and a thick large blood vessel surrounded by mild necrotic patch (low power, H & E stain).



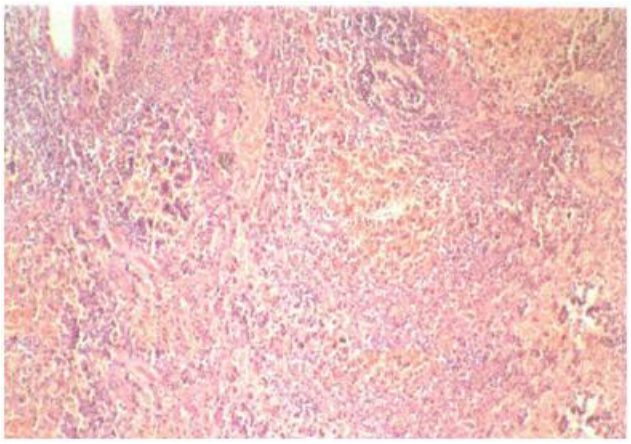
Photomicrograph 4b. Section of mice lung inoculated with L monocytogenes NCTC 7973 (passaged). Picture showing large necrotic patch. A granulomatous like pictures is distinct (high power, H & E stain).



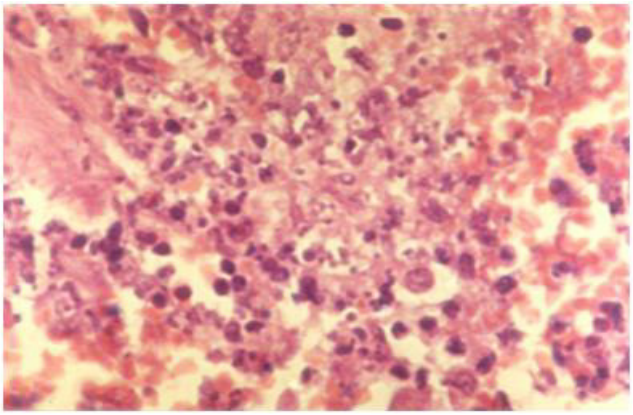
Photomicrograph 5a. Section of mice liver inoculated with L monocytogenes NCTC 7973 (unpassaged). Picture showing distinct necrotic areas, with loss of lobular architecture (low power H & E stain).



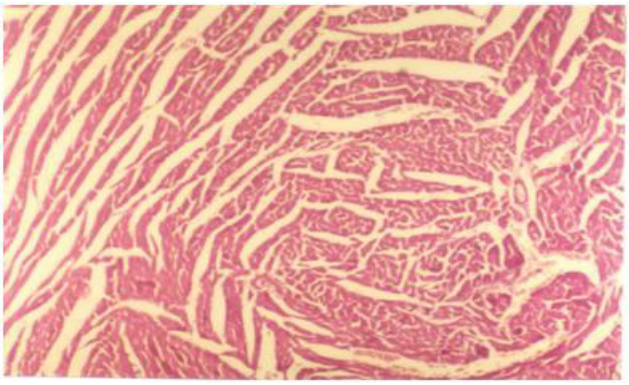
Photomicrograph 5b. Section of mice liver inoculated with L monocytogenes NCTC 7973 (unpassaged). Picture showing necrotic foci, a microabscesses, with destruction of liver parenchyma, numerous inflammatory cells and neutrophils are seen (high power, H & E stain).



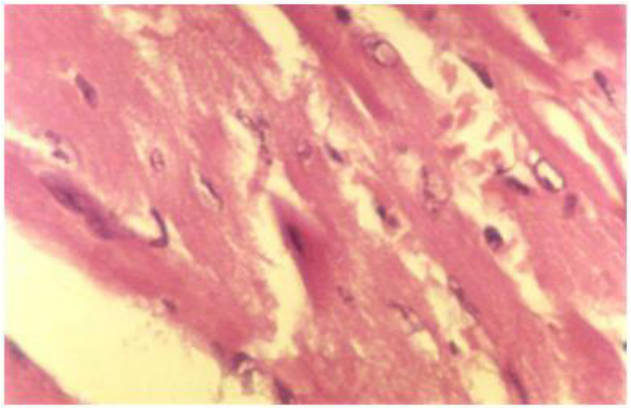
Photomicrograph 6a. Section of mice spleen inoculated with L monocytogenes NCTC
7973 un(passaged). Picture showing red pulp and white pulp area, mild necrosis and loss of normal spleenic architecture (low power, H & E stain).



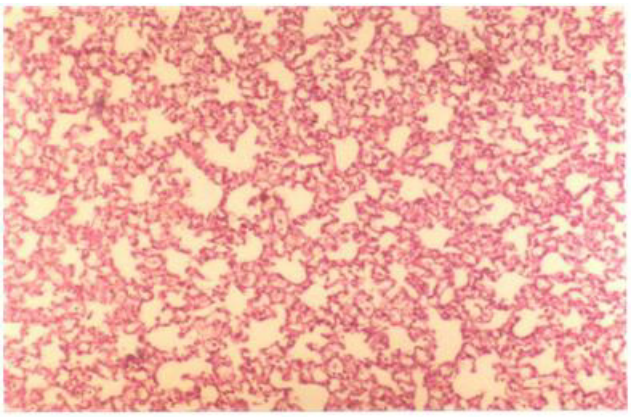
Photomicrograph 6b. Section of mice spleen inoculated with L monocytogenes NCTC
7973 un(passaged). Picture showing inflammatory cells, and destruction of spleen strcture, red cells are seen (high power, H & E).



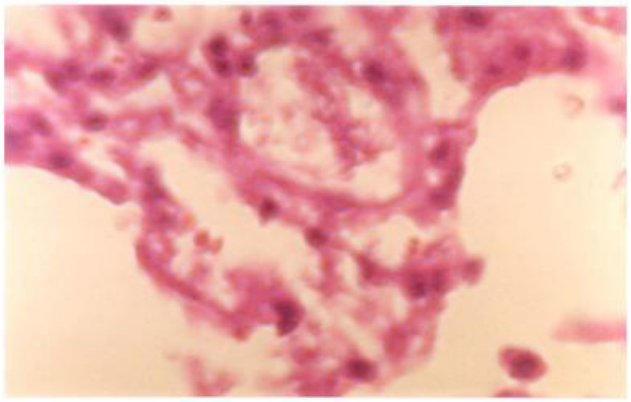
Photomicrograph 7a. Section of mice heart inoculated with L monocytogenes NCTC
7973 un(passaged). Picture showing mild inflammatory cells, loss of straitation and necrosis (low power, H & E stain).



Photomicrograph 7b. Section of mice heart inoculated with L monocytogenes NCTC
7973 un(passaged). Picture showing mild necrotic areas with inflammatory changes with loss of normal architecture (high power, H & E stain).



Photomicrograph 8a. Section of mice lung inoculated with L monocytogenes NCTC
7973 un(passaged). Picture showing area of mild necrotic along with numerous normal looking alveoli and minute capillaries (low power, H & E stain).



Photomicrograph 8b. Section of mice lung inoculated with L monocytogenes NCTC
7973 un(passaged). Picture showing large area of necrosis infiltrated by inflammatory cell and thick alveolar walls (high power , H & E stain).

not severe as in case of liver, where a ganulomatous like picture is seen (Photomicrograph 4a, 4b).

The liver of control rats revealed that hepatocytes, portal triads and vasculature appeared within normal limit (25). Lesions caused by the *L. monocytogenes* NCTC 7973 (unpassaged) culture in liver and spleen were low in intensity and the number of necrotic foci were less, containing no degenerating leukocytes and cellular debris from hepatocytes (Photomicrograph 5a, 5b, 6a, 6b resulting in the loss of normal lobular architecture. Similarly, heart and lung of the same mice show mild inflammatory response as compared the passage culture (Photomicrograph 7a, 7b, 8a, 8b).

DISCUSSION

Pathogenesis by *Listeria* is a process affected by many bacterial factors. The intracellular pathogen multiplies in the cytoplasm of the host cell after escaping from the phagosome formed either after direct internalization or after cell to cell spread (Brosch et al., 1992).

Animal passging increased the virulence of the culture by 10 fold. The difference in the pathogenicity of passaged and unpassaged is substantiated by the histopathological findings. The present data confirms the earlier report (Khan et al., 1973) that passage and unpassaged *L. Monocytogenes* cells show striking differences in their ability to cause lesions in the lymphoid organs after i.p. inoculation. The present results obtained fall in lines with the studies of Siddiqui (Siddidque, 1978) and Martin (Martin and Ehlers, 1995) who observed characteristic histopathological lesions of the liver in *Listeria* infected animals. The liver is responsible for metabolism and detoxification of the most of components that enter the body (Siddidque, 1978). Similarly, in present study, *Listeria* induced tissue lesions ranged from foci of necrosis without any or with only lesser inflammatory reaction, over abscesses of various sizes to granulomas without giant cells. In case of *L. monocytogenes* infected mice, large numbers of mononuclear cells were seen at the periphery of the necrotic focus, bacteria may pass from one host cell to another without transversing the interstitial space. A similar process has been described in *Shiegella flexeneri* and *Escherichia coli* infections (Stanley et al., 1970; Siddidque, 1978).

In the present study *L. monocytogenes* (passaged and unpassaged) striking difference in their ability to cause lesions in the lymphoid organs after i.p. route which confirms previous findings of Audurier et al. (1981) and Marco et al. (1991).

Moreover, in the present work, it has also been experienced that the animal passage enhances virulence of the bacterium, confirmed by the histopathological findings. *L. monocytogenes* NCTC 7973 rabbit passage culture produced comparatively more pronounced lesions and hence more destruction, than unpassage *L. monocytogenes* NCTC 7973.

The low virulence of *Listeria (*unpassage) indirectly suggests that there would be a higher carrier rate among men and animals under natural conditions (Khan et al., 1973). It was observed that some of the low doses infected mice seemed to have established a host-parasite balance without showing symptoms of acute illness.

CONCLUSION

In conclusion, it can be argued that infection with sub lethal dose may provide protection. It seems that a major portion of the human population is protected as a result of Subclincal infection from natural sources of *L. monocytogenes*. This appears to be borne out by the experiments of Notermans et al. (1992) working on these lines, who showed that human individuals are protected from *Listeria* infection as they carry T-cells with specific reactivity to *Listeria* species.

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