Studies on

STAPHYLOCOCCAL FLORA IN MILK from APPARENTLY HEALTHY UDDERS

THESIS

Submitted to the

Magadh University

In Partial Fulfilment of the Requirements for the Degree of

M. Sc. (Vet.)

Chittaranjan Prasad

Post-Graduate Department of Bacteriology

BIHAR VETERINARY COLLEGE, PATNA

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Sri P.B.KUPPUSWAMY,
B.A.,G.M.V.C.,B.V.Sc.,P.G.(Muk.),
P.G.(N.Z.),M.S.(Missouri),
P R I N C I P A L, &
Head of the Department of
Pathology & Bacteriology,

BIHAR VETERINARY COLLEGE,
PATNA

December,1967.

This is to certify that the work embodied in the Thesis entitled "Studies on Staphylococcal Flora in Milk from Apparanelty Healthy Udders" was carried out by Sri Chittaranjan Prasad, a candidate for the degree of M.Sc.(Vet.) with Bacteriology as his major subject, under my guidance and supervision.

P.B. Kuppuswamy
(P. B. KUPPUSWAMY)
31/12/67

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A C K N O W L E D G M E N T S

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(CHITTARANJAN PRASAD)

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I N T R O D U C T I O N

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INTRODUCTION

The crying need at present is more food to feed the increasing population. The role of food stuff of animal origin in easing the acute food shortage cannot be overemphasized. So, it is necessary that we pay every attention in preventing losses in animal production.

Bovine mastitis is of great economic importance to the dairy industry. Exact figures of losses due to this dreadful disease, though not available, still estimates based on local surveys reveal considerable losses in terms of milk and also deterioration in the quality of milch animal.

In India, annual loss due to discarding of milk from clinical cases and involution of affected quarters due to mastitis was estimated to the tune of 105 million rupees annually leaving apart the reduction in milk yield due to subclinical mastitis (Dhanda and Sethi, 1962).

Staphylococci, though a well recognised problem in human medicine received not much attention of the Veterinary investigators except as being an important factor in the causation of mastitis in India (Dhanda and Sethi, loc.cit.). In Bihar alone, staphylococcus was considered to be occupying the predominant position over other etiological agents associated with bovine mastitis (Prasad and Prasad,1963).

The disease producing potentiality of this organism varies from mild abscess to serious septicaemic and pyemic condition sometimes fatal to the host. The common pathogenic conditions associated with this organism apart from mastitis are; pneumonia, pleuritis, suppurative peritonitis in sheep and goats, botryomycosis in horses, pustular dermatitis and 'bumble foot' in poultry, arthritis in geese and various other suppurative and inflammatory conditions in various species of animals and human beings.

The staphylococcal infection assumes a zoonotic problem due to its association with food poisoning after consumption of infected milk and milk products.

The staphylococci received attention by microbiologists, since the time it was demonstrated in pus from operation wound by great Alexander Ogston in the year 1880. With the epoch making discovery of penicillin by Alexander Fleming in 1923 and subsequent addition of various other chemotherapeutic agents an impression was created in the medical and veterinary world that the problem of staphylococci has not only been solved but the battle against this potential enemy has finally been won. But the powerful defensive mechanism in staphylococci in the form of penicillin destroying enzyme namely penicillinase shattered the hopes and so again it aroused interest among the research workers in this organism. The introduction of newer antibiotics was tried to combat the

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disquietening feature due to emergence of resistant strains but temporarily. The staphylococci had learnt to resist every antibiotic, so far introduced.

The ubiquitous nature and variable characters of staphylococci have baffled the attempts for establishing any suitable criterion as the definite character of pathogenicity. Fairly intensive work on the subject in the past two decades has not been able to solve the missing links in our concept of the pathogenicity and virulence of staphylococci.

The much studied yet, little understood behaviour of staphylococci has rightly been described by Barber (1947). He expressed "The staphylococci is so familiar to us that we are tempted to think that we understand its mode of attack. In fact, they are still baffling to medical science. We have learned much about its potentialities but, are still unable to forecast its effect on a given individual or community".

It is true that certain types of bacteria particularly nonhaemolytic, coagulase negative staphylococci inhabit in the milk cistern and larger milk ducts for definite period without causing clinical mastitis. But not much information is available on the incidence of pathogenic strains of staphylococci inhabiting the apparently healthy udders.

Mere presence of this organism in the milk is, of course, not the exclusive evidence to prove that it is pathogenic but its pathogenicity has to be established by

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bacteriological techniques usually employed to determine its pathogenicity or otherwise. It is with this object, the present studies have been undertaken to assess the actual incidence of pathogenic strains of staphylococci in apparently healthy udders.

Resistance to antimicrobial drugs has attracted intense interest as it is a serious problem in the treatment of diseases. Staphylococci occupy the premier position among the etiological agents of mastitis and the antibiotic sensitivity of the isolated organisms was, therefore, conducted to find out the extent of resistant strains in this group of organisms. Such a study will also throw light whether milk from apparently healthy udders carry any antibiotic resistant staphylococci which might be a public health hazard.

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R E V I E W
O F
L I T E R A T U R E

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REVIEW OF LITERATURE

GENERAL

In the early years of microbe hunting it was believed by even eminent workers like Roberts (1874) and Lister (1878) that milk within healthy udder was germ free and presence of bacteria in the freshly drawn milk was considered to be associated with diseased udders. But in the following decade numerous investigators observed that milk drawn aseptically from apparently healthy cows also contained bacteria (Shulz,1892; Moore, 1899 and Ward, 1900) and it was generally accepted that bovine udder is inhabited by a 'normal flora'.

Evans (1916) showed that staphylococci and streptococci formed the major part of the normal udder flora. Subsequent workers confirmed the high incidence of staphylococci among the bacterial flora in normal milk and studied its various biochemical characters (Kastli & Binz,1948; Shah & Laxminarayanan, 1949; Bovim, 1950; Chute et al., 1954 and Malek & Kirdani, 1956).

Reid & Wilson (1959) carried out a comparative study of the biochemical activities of staphylococci isolated from normal and diseased bovine udders and observed only slight differences in their properties.

With the development of normal udder flora theory, the relation of bacteria in udder secretion to mastitis was brought under investigation.

Franck (1875) produced typical mastitis by inserting the secretions of affected cows into the teat ducts of apparently normal animals. The indication that mastitis could be infectious, was rather, confirmed by the outstanding contribution of Nocard & Mollereau (1884) who successfully isolated the organisms from the secretion of cows affected with mastitis and produced the disease by injecting a culture of the organism into the milk cistern of a healthy cow. Guillebeau (1890) isolated staphylococci, streptococci and coliform organisms from clinical cases of mastitis. He held these organisms responsible for the udder inflammation.

By 1900, it was fairly established that staphylococci were frequently present in the milk drawn aseptically from clinically healthy udder and the association of this organism with mastitis.

The isolation of 'micrococci' in 52 out of 85 cases of mastitis by Guillebeau (loc.cit.) and his contention that these organisms were responsible for the severe "udder inflammation", attracted the attention of many workers. Several workers confirmed his view and thereby much importance was attached to staphylococci in the etiology of bovine mastitis (Jones, 1918; Carpenter, 1925; Hardenberg & Schottmuller,1927;

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Minett et al.,1929; Little & Foley, 1935; Minett,1937; Little & Plastridge, 1946 and Reid & Wilson, loc.cit.).

Incidence of mastitis in India:

Land in 1926 probably recorded the first incidence of mastitis in cattle in India. Numerous investigators later on reported the occurrence of the disease in cow, buffalo and even in goat (Menon, 1935; Naik,1936 and Bawa, 1938). Dutta (1939) reported isolation of Staphylococcus aureus from majority of the cases of mastitis examined by him. Kuppuswamy (1943) brought forward the incidence of streptococcal mastitis in a herd from Bihar. Narayanan and Iya (1953) observed that out of 617 quarters of cow examined by him nearly 35 percent were infected and haemolytic staphylococci were found in 58 percent of the infected quarters. Prasad et al.(1961) reported,staphylococci formed 91 percent of the infection among the infected quarters in his investigation of the different dairy farms in Bihar for the incidence of bovine mastitis.

Dhanda & Sethi (1962) summarised after analysing the results of investigation on the etiology of bovine mastitis that Staph.aureus and various species of streptococci were found responsible for about 98 percent of the total number of cases, the remaining being due to Corynebacterium pyogenes, Pseudomonas pyocyanae and yeasts.

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Public health importance of staphylococcal infections in animals:

The first attempt to correlate staphylococcal infection with human disease dates back to 1914 according to Zinn et al.(1961) when acute gastroenteritis in man due to consumption of infected milk was reported, though the significance of the observation was not appreciated at that time.

Again, recurrent gastroenteritis in a school population due to staphylococci was reported by Crabtree & Litterer in 1934. The source of the staphylococci was traced to be infected bovine mammary gland.

With the passage of time, reports of various workers indicated that staphylococci from udder was responsible for many incidents of food poisoning in human beings (Hauge, 1951 and Steede & Smith, 1954). The capacity to produce enterotoxin was recognised by many workers as a characteristic of several strains of Staph.aureus isolated from bovine mammary gland (Bell & Veliz, 1952 and Nakagawa, 1958).

Instances of food poisoning due to consumption of spray-dried skim milk powder was attributed to preformed staphylococcal enterotoxin, producing gastroenteritis in school children after the consumption of reconstituted milk in England (Anderson & Stone, 1955).

The cause of food poisoning due to consumption of cheese and cheddar was traced out due to coagulase positive,

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beta haemolytic staphylococci isolated from animals which supplied milk to the plant involved was reported by Hendricks et al.(1959).

The other aspect of bovine staphylococcal infection which merit concern is the potentiality of such organisms in causing disease in man. The reported evidence of transmission of staphylococcal infection between animals and man (Poole & Baker, 1966), and animals as carrier of staphylococci pathogenic for human being increases the magnitude of staphylococcal infection in animals from public health point of view.

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STAPHYLOCOCCI

History:

Staphylococci, a widely distributed organism in nature, leading saprophytic life though some having disease producing capacity was probably the 'micrococci' of Robert Koch (1878) observed by him in pus. This was cultivated in liquid medium by Pasteur (1880). He inoculated the culture in rabbits which produced abscess and recovered the same micrococci from experimental animals.

Sir Alexander Ogston in 1881 demonstrated that these organisms were constantly present in acute and chronic abscesses and carried on the detailed study of this organism. Because of the resemblance of this organism to a bunch of grapes in stained preparation, Ogston coined the name staphylococcus to this group of organism from the Greek word Staphule (= grapes).

It was, however, left to Rosenbach in 1884 to carry out the detailed study of staphylococci and he isolated the organism in pure culture. Based on the pigment production he divided them into two species Staph. aureus and Staph. albus to which Passet (1885) added another name Staph. citreus, though chromogenicity being a variable character, did not form the satisfactory basis of classification for long.

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Classification:

The aim of the bacteriologists largely influences his approach to classification of the micro-organisms. Topley & Wilson has very rightly expressed the intention of the medical bacteriologists that they are interested to divide the microorganisms into pathogens and nonpathogens as their interest lies in knowing the ways in which bacteria interfere with human health. Therefore, to them such division would override other considerations. While systematist's classification is based on keeping the microorganisms into general order of living creatures with the least regard to their disease producing abilities.

Indeed, the wide distribution of staphylococci in nature, of which only a small proportion are purely parasitic and pathogenic has largely influenced the classification. Most of the attempts in classifying this organism was directed towards a division into two main groups - Staphylococci, pathogenic to man and animals and the rest nonpathogenic strains.

Chromogenicity:

The colour of the growth attracted much attention of early investigators and this property of staphylococci formed the first basis of classification (Rosenbach,1884 and Passet, 1885). The production of aureus colour was taken

as an index of pathogenicity (Verneuil,1880). But as it became established that this character was frequently variable, pigment as a basis of classification proved to be dubious aid.

It was noted earlier by Lubinski (1894) that golden colour is accentuated on exposure to light while anaerobic conditions, heating and repteated cultivation on artificial media produced loss of chromogenicity. Courmont(1897) succeeded in growing white and golden variants from the same abscess and such variants could not be distinguished serologically by phage typing (Barber, 1955) or by any other means. The white variants were not necessarily less virulent, propagated the idea that pigment production and virulence vary independently (Barber,1942). The instability of this character was reported by Dudgeon (1908) following his recovery of Staph. albus from an experimental animal earlier inoculated with Staph. aureus.

In the year 1938, Japanese workers demonstrated that pigment could be improved by growing the organism in 33 percent milk agar. Incubation at 22°C than 37°C was favoured for more evident pigmentation (Fujita & Yoshioka, 1938).

Inconsistent relation was noted between pigment production with that of production of coagulase by several workers. Cowan (1938) studied the biological properties of 114 human strains which included 82 coagulase positive and rest were negative. In the former group he reported 71 were of golden, 9 were white and 2 yellow in colour, while the second

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group was constituted of 30 white and 2 yellow coloured strains. The study of 13 coagulase positive and 7 coagulase negative strains of animal origin were made up of 9 golden and 5 white in the former group and 4 golden and 3 white in the later group.

The 156 human coagulase positive strains of staphylococci revealed 136 Staph. aureus, 11 canary yellow, 3 Staph. citreus and 6 were Staph. albus (Christie & Keogh, 1940). Smith (1947) found 79 golden yellow, 29 white and 2 yellow in 110 pathogenic strains. The 63 nonpathogenic strains were made up of 17 golden yellow, 44 white and 2 yellow coloured strains. He also concluded that majority of pathogenic strains isolated from dog produced white pigment.

Golden yellow colour were also seldom demonstrated in some of the nonpathogenic staphylococci obtained from marine flora and associated with spoilage (Wood, 1952).

George et al.(1962) examined 4834 bovine milk samples in his investigation for Staphylococcal infection and isolated 746 coagulase positive strains of staphylococci. Of the 688 coagulase positive strains he found 305 golden, 165 creamy, 179 white and 39 orange in colour.

Biochemical reactions:

Successful utilisation of fermentation reaction mainly with gram negative bacilli made many workers enthusiastic to try this approach for the classification of

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staphylococci also. Gordon (1904) and Dudgeon (1908) came forward with a number of carbohydrates and claimed that pathogenic strains have some distinctive features on the basis of action on these carbohydrates. Glucose, lactose, maltose, mannitol, glycerol, sucrose, fructose and mannose were the sugars consistently fermented by staphylococci whereas raffinose, salicin and inulin gave variable results (Dudgeon, loc.cit. and Chapman & Stiles, 1940).

Amongst all sugars the fermentation of mannitol, trehlose and mannose could be correlated with coagulase production.

Dudgeon & Simpson (1928) found close correlation between mannitol fermentation and pigment production. He reported 94 percent Staph. aureus fermented mannitol while Staph. albus attacked this carbohydrate to the extent of 40 percent only.

Thorough investigation by Cowan (1938) revealed 81 out of 82 coagulase positive strains produced acid in mannitol while only 5 out of 32 coagulase negative strains were mannitol fermenter. Close correlation between coagulase production and mannitol fermentation was reported by Christie & Keogh (1940) who observed 100 percent correlation between these two properties of staphylococci. Sethi (1957) found majority of the coagulase positive strains attacked mannitol with formation of acid. George et al. (1962) reported that

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approximately 50 percent of the coagulase positive strains examined by them isolated from bovine milk fermented mannitol. Joshi & Dale (1963) observed close agreement between coagulase production and mannitol fermentation when experiment was carried out under anaerobic condition.

Hine (1922) and Knott (1947) claimed that there was best correlation in fermentation of mannitol and pathogenicity amongst all sugars. Thygeson (1938) shared the same view when his all toxigenic strains gave positive reaction with mannitol, though only a few nontoxigenic strains were also positive.

Mannitol fermentation was regarded by many workers as a reliable criterion of pathogenicity for staphylococci (Andrewes & Gordon, 1905-1906; Dudgeon, loc.cit., Julianelle, 1937; Chapman et al.,1938, and Malik,1959).

The ability to hydrolyse gelatin by the strains of staphylococci has also been investigated since early days (Gordon, 1904). Evans (1916) gave stress on this property for differentiating pathogenic from nonpathogenic strains of staphylococci. Cowan (1938) noted that majority of the coagulase positive aureus strains liquefied gelatin though liquefaction was also recorded with coagulase negative, white and yellow strains. Smith (1947) found that 106 out of 110 pathogenic strains liquefied gelatin and 36 out of 63 nonpathogenic strains were also gelatin liquefier. Evans & Niven (1950) reported that

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only 50 percent either toxigenic or nontoxigenic strains of staphylococci could hydrolyse gelatin. Elek & Levy (1950) and Reid & Wilson (1959) observed that 63 percent of 195 coagulase positive strains, 93 percent strains from acute mastitis cases, 65 percent from chronic bovine mastitis and 66 percent from normal bovine udders had capacity to liquefy gelatin.

Other biochemical reactions shown by staphylococci were not considered of much differential value.

The validity of biochemical reaction as the basis of classification was challenged by other workers (Winslow & Winslow, 1908 and Walker & Adkinson, 1917). Cumins (1913) considered the fermentation tests with staphylococci of no differential value. Wood (1950) went ahead in disfavouring the biochemical reactions as a criterion of classification and opined that even it was unusual for two colonies of a pure culture to show exactly the same biochemical tests. The reliability of this criterion was further doubted by the well known fact that the members of the subgroup II of genus Staphylococcus was coagulase negative yet capable of fermenting mannitol. Selbie and Simon (1952) found no correlation between gelatin liquefaction and virulence test in mice.

Conflicting account about various biochemical reactions fails to establish it as an absolutely reliable criterion and are considered of little value in the identification of pathogenic staphylococci.

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Coagulase activity:

Most of the workers interested in the biological study of staphylococci, by now, were busy in search of a single yet satisfactory criterion of pathogenicity. This important character was hunted to utilize it as a diagnostic adjunct in the clinical laboratory.

The power of clotting citrated or oxalated plasma by some cultures of staphylococci was noted long back in the year 1903 by Leo Loeb. He employed goose plasma in his study and concluded that this effect was probably due to an enzyme preformed in the culture.

Much (1908) first drew attention to the association of this property with pathogenicity. He also reported by using horse and human plasma that only the aureus strains and not albus or citreus were able to produce this effect.

The whole phenomenon was, however, largely neglected until Daranyi (1926) advocated this property as the characteristic of staphylococcal pathogenicity. His view was later, shared and confirmed by various workers from different parts of the world (Gross, 1927; Chapman et al.,1934; Fisher, 1936a; Cruickshank, 1937 and Fairbrother, 1940).

Ability of udder staphylococci to coagulate human plasma was reported by Plastridge et al.(1938) to be more closely related to evidence of mastitis than any other test used by him.

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Gradually, the usefulness of this property of staphylococci was universally appreciated when hundred percent or near correlation between coagulase production and pathogenicity was reported by numerous workers (Christei & Keogh,1940; Moss et al.,1941; Smith, 1947; Elek & Levy, 1950 and Smith & Johnstone, 1956).

Lack & Wailling (1954) regarded absence of coagulase production as a strong indication of nonpathogenicity and Barber (1960) concluded 'though a positive coagulase test is only a rough guide, which does not necessarily imply full pathogenicity and gives no indication of virulence.

Although coagulase was tacitly assumed to play an important role in the virulence of staphylococci and was taken as a dependable criterion in estimating the potential pathogenicity of strain, direct demonstration of its role in pathogenicity was demonstrated by Ekstedt and Yotis (1960). He demonstrated that coagulase negative haemolytic strains of staphylococci suspended in partially purified coagulase solution proved fatal for many mice while the organisms alone or coagulase alone were innocuous.

The diverse reactivity of the plasmas of various species of animals employed in coagulase test and also the same plasma but adopting different techniques of the test drew the attention of various workers.

Human and rabbit plasma were considered as most susceptible by a number of workers (Much, loc.cit.,

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Walston, 1935; Pijoan, 1935 and Cruickshank, 1937).

The guineapig plasma was reported to be resistant to coagulase action at 37°C but was found susceptible at 20°C (Venbrenseghem, 1934). Smith & Hale (1944) reported that the fowl and mouse plasma did not clot at all.

Klatt (1954) advocated human and rabbit plasma to be the best; plasmas from horse and pig to be less reliable and plasma from cattle unreliable for bovine mastitis strains. Nakagawa (1958) reported, after working with a large number of bovine udder strains that rabbit plasma gave best result while plasmas of horse and sheep were less susceptible and cow plasma, the least.

Malik (1959) found the reliability of plasma from horse, sheep, cow and buffalo in decreasing order in relation to rabbit plasma during his study with 138 bovine strains of staphylococci.

The difference in the behaviour of plasma from different species led the workers, engaged in this field, to think and explore the possibilities for the presence of coagulase inhibitor or absence of coagulase activator substances in the plasmas of the different species of animals.

Smith & Hale (loc.cit.) demonstrated the presence of an accessory factor essential for the reaction in the plasma and postulated that the absence of activator substance in inert plasmas prevented clotting.

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In the meantime, the observation of variable results with undiluted human plasma was reported which could not be accounted fully in terms of 'activator' substance. An inhibitor substance responsible for this folly was reported to be present in human plasma by several workers (Gross, 1933a ; Walston, 1935; Lominski and Roberts, 1946 and Kaplan & Spink, 1948). This inhibitor substance was also demonstrated in spleen extracts by Mercier et al.(1947).

Fresh plasma was advocated by Christie & Keogh (1940), while Fisk (1940) and Fairbrother (1940) reported good results with several week old citrated plasma kept at low temperature in sterile condition. Chapman (1944) considered the use of human whole blood better instead of plasma as he got earlier results with the former.

The aim of an ideal test for the study of coagulase activity be 'to detect the largest number of coagulase producing strains within a short time, without obtaining false positive results with morphologically similar cocci' (Elek, 1959).

The first method for the study of this phenomenon was that of Loeb's which was modified subsequently by several other workers (Daranyi, loc.cit., Chapman et al., 1934; Fisher,1936 and Fisk, 1940). This was popularly called tube method for the study of coagulase activity.

Penfold (1944) introduced plate method in which coagulase activity could be detected by drawing the

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organism on nutrient agar containing plasma. A zone of turbidity as a result of deposition of insoluble fibrin around the colonies of staphylococci was indicative of coagulase action. This technique was adopted by a number of workers to study the coagulase activity of staphylococci (Reid & Jackson, 1945; Smith, loc.cit., Iack & Wailling, loc.cit. and Malik, loc.cit.).

More recently, while Cadness-Graves et al. (1943) evolved slide technique, capillary method for estimation of coagulase activity of staphylococci was reported by Griffith & William (1959).

Alder et al.(1953) found when his 179 strains were subjected to test for coagulase activity, 121 gave positive result with tube test and 118 by slide technique. Malik(loc.cit.) utilised tube, plate and slide method to determine the coagulase activity of 100 bovine strains and found that 40, 39 and 34 strains respectively gave positive results using rabbit plasma.

The presence of coagulating substance in the filtrate was demonstrated by many workers. Fisher (loc.cit.) reported coagulase to be stable between 60°to 100°C depending on individual strain. The precipitation of the active principle of coagulase by alcohol, acetic acid or half saturated ammonium sulphate was also demonstrated (Walston,1935 and Fisher,loc.cit.). Coagulase was reported to be antigenic by several workers (Gross,1933; Walston,loc.cit.; Tager & Hales,1948 and Duthie, 1952).

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Duthie (1954) produced evidence of existence of two forms of coagulase - 'bound coagulase' or 'clumping factor' which was attached to the cells and was responsible for slide clumping and 'free coagulase' which was liberated into the medium and was responsible for the tube coagulase test. 'The bound coagulase' or 'clumping factor' acted directly on fibrinogen, while 'free coagulase' required the accessory factor.

The optimum condition for the production of coagulase by staphylococci was investigated by several workers without any conclusive result.

Attempts for the quantitative estimation of coagulase activity were made by Lack & Wailling (1954), Inniss & Sanclemente (1962) and various other workers.

Haemolysis:

The ability of staphylococci to produce haemolysins on blood agar plates was once regarded a criterion of pathogenicity.

Kraus & Clairmond (1900) was probably the first man to notice this phenomenon. Eikman (1901) introduced blood agar plates for the demonstration of haemolysin production. On the basis of susceptibility of the red cells of various species of animals towards the toxic filtrate and other characteristics, the different types of haemolysins were detected in staphylococci.

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Glenny and Stevens (1935) described two types of haemolysin produced by staphylococci and designated as alpha and beta toxins. The basis of differentiation was lethal, dermonecrotic and haemolytic properties on suitable laboratory animals along with neutralization with corresponding antitoxins. Further study of alpha toxigenic strains helped Morgan and Graydon (1936) to postulate the two components of alpha toxin- $alpha_1$ and $alpha_2$, to which the existence of third fraction was later added by Kodam & Kojima (1939) and called $alpha_3$.

Smith & Price (1938) reported the presence of another haemolysin which was different from both alpha and beta lysin serologically and in other aspects. They possessed the ability of lysing the red cells of a number of species of animals including man. This was called gama haemolysin. Yet, another member designated as delta haemolysin was added by Williams & Harper (1947). This lysin was haemolytic to a variety of mammalian species' blood including equines. This was differentiated from alpha and beta toxin on plate containing an excess of alpha and beta antitoxin.

Thaysen (1948) reported two antigenically different fractions of beta lysin -$beta_1$ and $beta_2$. Elek & Levy (1950) came forward with evidence of a new haemolysin namely epsilon produced by a coagulase negative strain of staphylococci. It was capable of haemolysing sheep and rabbit blood cells and was not inhibited by staphylococcal antitoxin.

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Kutscher & Konrich (1904) stated that all pyogenic staphylococci produce haemolysin. Jullianele (1922) found haemolytic substance in the broth culture of his coagulase positive staphylococci isolated from human origin. This property was appreciated by Daranyi (1929) in playing a role in pathogenicity. He reported some coagulase negative strains also share this property.

The report of Cowan (1938) indicated the presence of alpha, beta and their combination in all coagulase positive strains from human and animal origin. The coagulase negative strains examined by him were all nonhaemolytic. Similar findings were reported later by Christie et al.(1946). On the other hand, Williams & Harper (1946) found only 31 percent and Jackson et al.(1955) reported only 82 percent of coagulase positive strains which produced alpha lysin.

The observation of Daranyi(loc.cit.) that some coagulase negative strains also produce heamolysin was confirmed by Keogh (1940) when he found haemolysin production in coagulase negative strains also. Joshi & Dale (1963) compared the haemolytic activity and coagulase production of staphylococci from udder. They found 94 percent of the 154 coagulase positive strains were haemolytic while of 24 coagulase negative strains only 71 percent were haemolytic.

The production of haemolysin by staphylococci was considered a dependable criterion of pathogenicity by several

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workers but Boe (1944), Smith (1947) and Lack & Wailling (1954) attached little importance to this property with the association of pathogenicity. Schalm & Woods (1953), Slanetz & Bartley (1953) and Nakagawa (1958) demonstrated the coagulase negative haemolytic strains of staphylococci in bovine udder and considered them nonpathogenic.

The close correlation between haemolysin production and coagulase activity did not necessarily indicate that it played some biological role in the evolution of lesions. In actual practice, no correlation was demonstrated between the amount of alpha lysin production and severity of clinical condition (Dolman,1932; Tager & Hales, 1947).

The study of alpha and beta haemolysins were made by Bryce & Rountree (1936), Smith & Price (1938a) and Flaum (1938). They discovered that alpha lysin had lethal effect on rabbit and mice, haemolytic effect on rabbit and sheep erythrocytes at 37°C and dermonecrotic lesion was recorded on guineapigs and rabbits, while beta lysin was lethal for rabbits only, had haemolytic effect on sheep and human erythrocytes when brought to lower temperature but failed to produce dermonecrotic effect.

Fulton (1943) reported about the behaviour of alpha lysin towards heat that at lower temperature (65°C) the toxin was inactivated due to union of the active agent with protein of the disintegrated organisms or of the media, which

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was however destroyed at higher temperature (100°C) liberating free toxin.

The production of beta toxin was considered characteristic of animal strain by Minett (1936). His view was later shared by various other workers (Bryce & Rountree,1936; Smith, 1947; Elek & Levy, 1950; Saxena, 1954 and Nakagawa,1958). The relation of alpha toxin production to human strain was advocated by many workers (Bryce & Rountree, loc.cit., Cowan, 1938; Christie & Keogh, 1940 and Christie et al.,1946).

Williams & Harper (1947) described the method of differentiating alpha, beta and delta haemolysins by the use of rabbit, sheep and horse red cells by plate and tube techniques. The identification of different types of haemolysins by the use of antitoxin was also recommended by Gillespie & Simpson,(1948)and Elek & Levy,(loc.cit.).

The exploration about the production of haemolysin in staphylococci by many investigators revealed many interesting facts about it.

Walbum (1922) found the incorporation of magnesium, nickel, manganese, gold and platinum salts in the medium have stimulatory effect on the production of haemolysin, whereas salts of calcium retarded its production. The salutory effect of carbon dioxide was recognised by Parker et al.(1925) on the production of haemolysin. [illegible] al effect of carb[illegible] ted

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the alkaline shift of the medium, so harmful for the lysin production. Parish and Clark (1932) reported a medium comprising of 4 percent peptone buffer broth with a base of 0.8 percent agar for the good yield of toxin at an atmosphere of 25 percent carbondioxide. But McClean (1937) did not consider the presence of agar essential for toxin production and concluded that substances like chopped up cellophane, kaoline or calcium phosphate could replace agar in the medium.

Animal pathogenicity:

Like other studies on the pathogenicity of staphylococci, various laboratory animals were used to test the pathogenicity of this organism. As per the opinion expressed by Miles (1955) that actually the virulence of an organism could only be defined properly as to its disease producing capacity in relation to certain susceptible host, various species of laboratory animals were tried to carry out the test.

Rabbits were frequently used by several workers to study the lethal, dermonecrotic and haemolytic effect. Intradermal, intravenous and subcutaneous routes were utilised for inoculation (Minett,1936; Cruickshank,1937 and Flaum, 1938).

The test of pathogenicity of staphylococci was conducted on mice by Minett (1936). He inoculated 66 mice

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with 0.1 cc. of 24 hrs. broth culture of 33 strains of staphylococci, using two mice for each strain and observed that 19 mice died in the course of 7 days after inoculation. Pisu (1951) considered mice inoculation as most reliable test of pathogenicity. Intramuscular route in mice was utilized to study the pathogenicity by Selbie & Simon (1952). They inoculated 0.2 ml. of 18 hrs. broth culture at the back of the thigh which led to the production of local lesion and increased in size upto about 4th day. They estimated virulence by measuring the increase in girth and found that this was correlated with alpha lysin, coagulase and fibrinolysin production.

Pannisett & Dobija (1953) suggested intra-cranial route of inoculation in mice with broth cultures which was successfully emplyed by Ekstedt and Yotis (1960) to study the effect of coagulase on the coagulase negative strains of staphylococci for the increase of virulence. The virulence of different strains of staphylococci were tested by Bass & Higginebothams (1960) on mice by using intravenous and intra-peritoneal routes and also with or without the addition of mucin to the inoculum. They concluded that no single method could be advocated for assessment of virulence,as a strain proved virulent by one method was not so when other method was employed.

While,Schneierson et al.(1961) used intra-peritoneal route in mice Gerhardt et al.(1962) studied the pathogenicity of a human strain of Staph. aureus in mice by intramuscular inoculation.

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Chick embryo, guineapigs, pegions and hamstrings were reported to be used as experimental animals for detection of pathogenicity of staphylococci.

Antibiotic sensitivity:

When penicillin was added in the clinician's armament, the majority of staphylococci were sensitive to it (Abraham et al.,1941). But with the passage of time and increased use of antibiotics, it was brought to notice that the acclaimed penicillin was found ineffective in some of the staphylococcal infections.

The insensitive strains of staphylococci were occasionally encountered by Fleming (1942) himself. Similar observation was also made by Hobby et al.(1942), though it could not catch the mind of these workers. Later on, this problem of resistance attracted many investigators engaged in this field.

Penicillin resistant strains of staphylococci were demonstrated to be many and varied. According to their response to the antibiotic they may be divided into three groups: viz., drug tolerant, drug dependent and drug destructive. Organisms of the first group are capable of growing in the presence of an increased concentration of antibiotic, which remains unchanged and retains full bactericidal effect for other bacteria; the growth of the second group of organisms are

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favoured or even completely dependent on the presence of the antibiotic but organisms of the third group produce an antagonist substance which inactivates the antibiotic. Penicillin tolerant and penicillin dependent strains occur in in vitro studies while penicillin resistant strains are cause of penicillin resistant staphylococcal infections (Barber,1957).

The mechanism of penicillin resistant strains is not fully understood, yet most workers held the penicillinase, an enzyme produced by resistant strains responsible for the inactivation of penicillin (Barber, loc.cit.).

Penicillinase production by the resistant strains of staphylococci attracted many investigators to work in this field in the subsequent years (Kirby, 1945; Gilson & Parker, 1948 and Eriksen & Hansen, 1954).

Enzyme adaptation, selection and spontaneous mutation were attributed as the possible mode of origin of penicillin resistant strains of staphylococci.

With regard to the correlation between penicillin susceptibility and virulence, Amsterdam & Schneierson (1957) reported on 80 coagulase positive, haemolytic and mannitol fermenting strains of staphylococci. They observed considerable variation in the pathogenicity for mice between the resistant and sensitive strains. The former proved more virulent in significantly higher proportion. No definite pattern in respect of change in virulence was observed by them in artificially induced resistant strains.

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Though much energy has been invested in surveying the penicillin resistant strains of staphylococci by the workers interested in human medicine and they have realised the magnitude of the menace to a great extent, the perusal of the available literature indicates that no concerted move has been undertaken by the workers of veterinary profession. With the advancement of animal industry and increased use of antibiotics in the treatment of animal diseases, this aspect is gaining importance.

Murnane (1945) reported that all the strains of staphylococci isolated from clinical cases of mastitis were inhibited by 0.1 unit/ml. of penicillin and could find no evidence of acquired tolerance in staphylococci as a result of repeated treatment. The study on penicillin susceptibility of the staphylococci by Schalm & Woods (1953) isolated from the two herds revealed that percentage of resistant strains vary between two herds. In one herd they found that 56 percent of the strains grew in presence of 1 unit of penicillin and 18 percent in 10 units while the corresponding percentage of the second herd was only 4 and 1.5 respectively.

Aynsley (1953) examined 500 cultures from cases of bovine mastitis using ditch plate technique, the ditch containing 2 units/ml. of penicillin and reported that 14 cultures of staphylococci were resistant to penicillin. This group provided the largest number of resistant organisms. The

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inhibiting concentration of penicillin fell between 0.02 and 5.0 units/ml. when these 14 strains were tested by serial dilution method.

Penicillin sensitivity of staphylococci was also studied by Das (1958), Malik (1959) and Panduranga Rao et al.(1966). The number of workers is increasing with the time, who gets attracted to this problem.

Barber (1960) reported that the staphylococci developed resistance to streptomycin very rapidly. Resistance to tetracycline appear rather slowly (Pain et al.,1948).

The staphylococci, which were originally reported to have developed resistance to first member of the antibiotic group of drugs, soon learned to become resistant to subsequent antibiotics so far introduced.

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M A T E R I A L S
A N D
M E T H O D S

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MATERIALS AND METHODS

Milk samples collected from the cows of the Government Cattle Farm, Patna, constituted the materials in the present investigation. Milk was collected from healthy udders and none of the animals was suffering from mastitis, clinically at the time of collection, which was judged by physical examination of the udder and visual examination of milk samples.

Collection of materials:

Milk samples were collected under all possible sterile precautions to avoid any external contamination. The udder of each cow was washed with weak solution of potassium permanganate as routine practice followed in the farm and was wiped dry with a clean towel. The surface of the teat and its orifice were cleaned with a pedget of cotton soaked in 70 percent alcohol. The hand and fingers of the milker were also washed thoroughly with 10 percent carbolic acid soap and water followed by potassium permanganate solution after examining them for any wound etc. About 10 ml. of milk were collected in sterile tubes from each of the four quarters of each cow separately after discarding the first few streams.

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Isolation and detection of strains:

The procedure adopted for the isolation and detection of strains have been outlined in scheme on page 35. The term 'strain' has been used only to indicate isolates from different animals without implying any significant difference in their biological behaviour.

As depicted in the scheme the samples after transferring into sterile pyrex glass tubes in about 5 cc. quantities were centrifuged at 3000 r.p.m. for 10 minutes. The sediment and small amount of cream were streaked on blood agar medium containing 10 percent defibrinated bovine blood. Incubation was done for 24 hours at 37°C. The plates were examined after incubation for the growth of organisms. The growth of staphylococci was readily recognised by the characteristic colony characters.

Staph-110 medium was tried but was abandoned due to the inherent difficulty that it used to remain moist even after prolonged drying, inviting contamination and making isolation difficult. Two samples were streaked on each plate.

Primary identification of staphylococci was done by studying the colony characters, staining of the organisms with standard Gram's stain and catalase test. In some cases purification of culture was done by further plating. Further identification was done on the basis of growth and

Scheme showing the procedure for the isolation and detection of strains.

Materials to be examined

(Milk)

I

Centrifugation at 3000 r.p.m. for 10 minutes

I

Streaking of the sediment and a small quantity of cream on blood agar medium

I

Colony characteristics	Catalase test	Examination of Gram's stained smear

Examination of nutrient broth culture's smear (Gram's method)	Anaerobic fermentation of glucose.

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production of acid from glucose anaerobically in a peptone yeast extract medium containing Bromocresol purple as an indicator of acid production and examination of smears from 24 hours incubated broth culture stained by Gram's method.

Only those characteristic colonies which were catalase positive and fermented glucose anaerobically, having characteristic morphology in smears from broth culture stained by Gram's method were preserved on agar slants in the refrigerator, for further studies. The isolates were maintained by subculturing at an interval of one month.

A. Qualitative studies of physiological characters

Chromogenesis:

Isolated cultures were grown on nutrient agar slants at 37°C for 24 hours followed by 48 hours at room temperature. The colour of the growth (golden yellow, white and yellow) was observed and recorded.

Coagulase production:

The production of coagulase by strains of staphylococci has long been considered as an index for presumptive pathogenicity.

The test for coagulase activity was performed by mixing 5 drops of overnight culture in nutrient broth to

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0.5 ml. of rabbit plasma diluted with nutrient broth in the proportion of 1 in 10 (Cruickshank, 1965). All the isolated strains of staphylococci were also tested for coagulase activity employing human plasma instead of rabbit plasma. The inoculated tubes were incubated at 37°C for 4 hours. The result of the negative tubes were recorded after keeping them at room temperature,overnight. A known positive and negative controls were included with each day's test.

The results were recorded on the following criteria, as used by Malik (1959):-

Positive	-	Good clot with a little unclotted plasma.
Negative	-	No clot or plasma having granular appearance.

Before performing the tube test, the coagulase production of each strain was performed by employing slide technique using rabbit plasma described by Cadness-Graves et al.(1943).

Rabbit blood was collected by cardiopuncture in sterile tubes containing 4 percent citrated mixture. One ml. of 4 percent citrated mixture was used for every 10 ml. of blood and were mixed uniformly. The tubes were centrifuged at 1500 r.p.m. for 30 minutes and the separated plasma was transferred to sterile test tubes and stored in refrigerator.

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Human plasma was procured from the Blood bank of Patna Medical College Hospital, Patna.

Fermentation of carbohydrates:

The fermentative activity of the isolates were tested by employing two sugars, lactose and mannitol.

The medium comprised of peptone water incorporated with 1 percent of each sugar and Andrade's indicator. The organisms were inoculated in each of the sugar medium and incubated at 37°C aerobically for 72 hours.

Appearance of pink colour in the inoculated tubes was taken as indicator of fermentation.

Gelatin liquefaction:

The ability to liquefy gelatin was examined by growing the test strains in 15 percent gelatin medium and were incubated at 37°C for 14 days. To prevent evaporation of the medium, the tubes were plugged with cotton dipped in paraffin.

The result was read after keeping the incubated tubes in refrigerator for half an hour.

A positive and negative control were included with each day's test in order to detect the discrepancy, if any, in reading the result.

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Methyl-red and Voges-Proskauer test:

The test strains were grown in 5 cc. of glucose phosphate peptone water medium for 72 hours. 5 drops of methyl red indicator (0.04%) was added to culture. Appearance of red colour was indicative of positive result whereas yellow colour indicated negative result.

The Voges-Proskauer test was performed by adding about 1 ml. of 40 percent caustic potash solution to 72 hours grown culture of the test strain in the glucose phosphate peptone water medium and was then left at room temperature overnight. Development of brick red or pink colour was taken as positive and no colouration or slightly yellow colouration was considered as negative.

Nitrate reduction:

The test strain was grown in plain broth containing 2 percent potassium nitrate for 48 hours at 37°C. The presence of nitrite was tested by adding a few drops of 'Test solution-A' (containing 8 gms. of 0.5 percent sulphanilic acid in dilute sulphuric acid) and 'Test solution-B' (containing 6 ml. of dimethyl alphanaphthylamine in 1 litre of acetic acid) successively to about 2 ml. of the culture. Presence of red colour denoted the presence of nitrite.

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This method was adopted after Wallace and Neave (1927).

Salt tolerance:

The property of the isolated strains to grow in the presence of higher concentration of sodium chloride was examined by growing the strains in nutrient broth containing 10 percent common salt for 48 hours. Growth of the organism in the medium as was taken as positive.

Haemolytic activity:

The method followed to study the haemolysin production by the isolated strains was that of Williams and Harper (1947). The whole blood was used instead of red blood cells. Primary study on haemolysis was done by growing each strain on blood agar medium containing 5 percent defibrinated sheep blood for 48 hours at 37°C. Each haemolytic strain was later, streaked on separate blood agar plates containing 5 percent defibrinated sheep, rabbit or horse blood. The streaked plates were incubated at 37°C in the presence of 30 percent carbondioxide tension for 48 hours.

A large zone of haemolysis with hazy margin on both rabbit and sheep agar plates was indicative of alpha haemolysis. The zone of haemolysis was wider on sheep blood

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agar plate than on rabbit blood agar plate.

Beta haemolysis was recorded by observing a wider zone of darkened red cells on sheep blood agar plate only. The darkened zone of haemolysis upon standing in refrigerator demonstrated 'hot-cold' lysis.

The small yet sharp zone of haemolysis on all the three agar plates was denoting the menifestation of delta haemolysin.

The results of haemolysis thus obtained by the above method were confirmed by Elek & Levy's (1950) technique. The staphylococcal antitoxin was kindly supplied by M/S Burroughs Wellcome & Co.

B. ANTIBIOTICS SENSITIVITY TEST

Antibiotic sensitivity of the isolated strains was conducted by serial plate dilution technique. This method was advocated as a better method than the use of antibiogram (Malik, 1960). The method suggested by Frisk (1945) and Erlarson (1951) was followed.

Nutrient agar medium was used as a basal medium in which antibiotics detailed below were incorporated to give the following concentration per ml. of the basal medium by serial dilution technique. Sterile distilled water was used

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as diluent instead of commonly used normal saline solution.

Name of the antibiotics	Concentration per ml. of the basal medium.
Penicillin	0.05 I.U.- 0.1 I.U.- 0.25 I.U.
	0.5 I.U. - 1.0 I.U.- 1.5 I.U.
	15 I.U. - 150 I.U. - 1500 I.U.
Streptomycin	10 micrograms & 15 micrograms
Terramycin (Oxytetracycline hydrochloride)	10 micrograms & 15 micrograms

Each test strain was grown in nutrient broth for 24 hours and one loopful of the nutrient broth culture was streaked on the each set of media containing antibiotics. One strain of E.coli was included to serve as positive control which was known resistant. The streaked plates were incubated for 24 hours at 37°C.

Complete inhibition of growth was taken as index of sensitivity.

C. MICE PATHOGENICITY TEST

Two healthy Swiss albino mice of about six weeks age weighing between 18-22 gms were inoculated intraperitoneally with 0.4 cc. of eighteen hours broth culture of each isolates. The mice were observed for eight days. Those which

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died within this period were necropsised and the lesions were observed. Attempts were also made to recover the organisms from the heart blood, spleen, kidney, liver and lungs. The rest were sacrificed on eighth day and isolation of organism was tried from the heart blood on blood agar medium. Two mice inoculated with simple broth and two noninoculated were also included in each group as control.

The isolates, which produced death of the mice and the recovery of organism from the heart blood of dead as well as sacrificed animals were regarded as pathogenic while others were taken as nonpathogenic.

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R E S U L T S
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RESULTS AND DISCUSSION

During the course of the present study, it was endeavoured to isolate staphylococci from the bovine milk from apparently normal udder. Possible care was taken to prevent extraneous contamination. The organisms, so isolated were studied for different cultural and biochemical characters. Pathogenicity test of the representative strains sorted out on the basis of certain biochemical reactions and antibiotic sensitivity was carried out on mice. The susceptibility of the isolated strains to commonly used antibiotics was also examined.

Incidence of staphylococci in normal udder:

Systematic bacteriological examination of 371 quarter samples of milk collected from 94 cows, resulted in the isolation of 135 strains of staphylococci.

TABLE - I

No. of staph. isolated	No. of quarters	Percentage of quarters affected.
135	371	36.3

Analysis of the results of the bacteriological examination as depicted in table-I reveals that 36.3 percent of the quarters yielded different strains of staphylococci.

Typing of isolated staphylococci on the basis of coagulase production resulted in differentiating the total strains into 48 coagulase positive strains and were taken as Staphylococcus aureus. Among the rest coagulase negative strains, 4 were identified as Staphylococcus citreus based on the production of yellow colour while the rest were considered Staphylococcus albus irrespective of white or golden yellow colour. The number of different types along with percentage has been given in table - II.

TABLE - II

Results of typing of staphylococci

Type of organism	No. of isolates	Percentage
Staph.aureus	48	35.55
Staph.albus	83	61.48
Staph. citreus	4	2.96

From the above table it appears that Staphylococcus aureus formed sizable portion of the isolates

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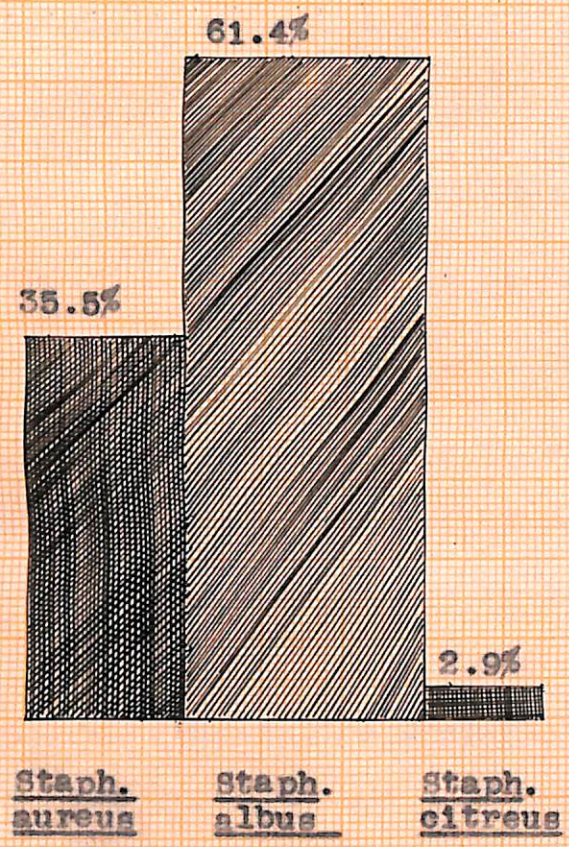
Percentage of Staphylococcal species.



being 35.5 percent. The Staphylococcus albus formed the bulk (61.4%) and Staphylococcus citreus was in lowest percentage, (2.9%).

The result of subclinical infection, considering the coagulase positive strains of staphylococci as potential pathogen, has been depicted in table-III.

TABLE-III

No.of coagulase positive strain.	No.of cows examined	Percentage of subclinical infection.	No.of quarters examined	Percentage of subclinical infection.
48	94	51.06	371	12.93

Perusal of the above table indicates that 51.06 percent of the animals examined were having subclinical udder infection and the percentage in terms of infected quarter was about 13%.

During the present investigation, the coagulase positive strains have been designated as Staph.aureus regardless of pigment production, Staph.citreus denotes the yellow coloured strains on the basis of pigment production and the rest coagulase negative strains have been called Staph. albus. It, thus, becomes evident that the modern approach in classifying the strains on coagulase status has been tried

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partially though chromogenicity as basis of classification has not been ignored also. The species of Staph.aureus contains organisms that produce phosphatase and coagulase, and utilize mannitol anaerobically (Biard-Parker,1965). The terminology aureus, albus and citreus on the basis of pigment production have been coined by previous workers (Rosenbach,1884 and Passet, 1885). The conflicting basis for classification is admitted, though such overlapping to a considerable extent may be expected till uniform criteria become acceptable to all bacteriologists.

The coagulase status has been regarded as a criterion for the identification of potential pathogens in the present investigation. Coagulase activity of staphylococci was advocated as an index of pathogenicity by Daranyi (1926). Subsequently several workers confirmed the usefulness of this property of staphylococci in the identification of potential pathogens,(Smith,1947; Smith & Dubos, 1956 and others). The role of coagulase in the pathogenesis of infection was demonstrated on experimental animals by Cohn & Morse (1959). They reported that coagulase positive staphylococci resisted phagocytosis, whereas coagulase negative strains were more readily digested and destroyed by the leucocytes of rabbits in in vitro condition, establishing the fact that coagulase might help in the establishment of infection by preventing elimination of the cocci. Ekstedt & Yotis (1960) had reported about the increase in the virulence of haemolytic,coagulase negative

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strains due to partially purified coagulase. Indirect support was given to this property as an index of pathogenicity by Lack & Wailling (1954) who regarded absence of coagulase production as a strong indication of nonpathogenicity and Barber (1960) considered a positive coagulase test as only a rough guide which did not necessarily imply full pathogenicity and as an indicator of virulence. However, the coagulase status of staphylococci has been regarded as an index of pathogenicity in the present studies seems justified keeping in view the report of the above workers.

The high incidence to the extent of 51 percent of staphylococcal colonisation of bovine udders, observed in this study can be favourably compared with that reported by other investigators. Shah & Laxminarayanan (1949) obtained 19 isolates in an examination of the udders of 50 cows. Nag & Prasad (1965) reported the incidence upto 35.5 percent in the dairy cows of Bihar.

In the present investigation Staph.aureus was recovered from 12.9 percent of the milk samples examined. The report of George et al.(1962) that examination of 4838 quarter samples of milk resulted in the isolation of 746 coagulase positive staphylococci,which formed about 15 percent of the samples examined by them; is in agreement with the finding of the present study. A higher percentage reported by Evans (1916) to the extent of 51.4 and 41.2 percent by Dhanda & Sethi (1962)

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was probably due to chromogenicity being the criterion for the identification of staphylococci. Other reasons for the fluctuating data on the incidence of Staph. aureus may be due to difference in the managerial conditions of the herd, numbers of milk samples examined and the difference in the condition of udders from which the milk was collected.

Physiological characters of isolated staphylococci strains:

Various physiological characters of the isolated strains of staphylococci were studied. The characters included chromogenicity, fermentation of lactose and mannitol, capacity to liquefy gelatin, reduction of nitrate, salt tolerance, M.R. and V.P.reactions. The haemolysin production and coagulase activity of the isolated strains were also examined.

Results of various physiological characters studied have been tabulated in table - IV.

Chromogenicity:

From the table-IV it appears that out of the total 135 strains, 62 (45.9%) were golden yellow, 69 (51.1%) white and 4 (2.9%) yellow in colour. The table-IV also reveals the distribution of various coloured strains in the two groups viz., coagulase positive and coagulase negative. 48 strains of

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TABLE - IV

Physiological and biochemical properties of staphylococci.

No. of strains	Colour			Strains showing positive reaction for					
	Golden	White	Yellow	M.R.	V.P.	Gelatin liq.	Salt tol.	Nitrate reduct.	Mannitol fermentation
Total 135	62	69	4	94	69	45	130	125	60
Percent.	45.9	51.1	2.9	69.6	51.1	33.3	96.2	92.6	44.4
Co.+ 48	21	27	-	45	37	12	48	48	44
Percent.	43.7	56.2	-	93.3	77.0	25.0	100	100	91.6
Co.- 87	41	42	4	49	32	33	82	77	16
Percent.	47.1	48.2	4.5	56.3	36.7	37.9	94.2	85.5	18.3

Co.+ = coagulase positive

Co.- = coagulase negative.

coagulase positive group comprised of 21 (43.7%) golden yellow and 27 (56.2%) white coloured strains while 87 coagulase negative strains consisted of 41 (47.1%) golden yellow, 42 (48.2%) white and 4 (4.5%) yellow coloured strains.

Fermentation of sugar:

The strains were tested for the production of acid in mannitol and lactose. Out of 135 strains, 60 (44.4%) produced acid in mannitol and 129 (95.5%) attacked lactose with the formation of acid. Distribution of mannitol and lactose fermenting strains among the coagulase positive and coagulase negative strains as shown in the table IV,indicated that all strains of the former group fermented lactose while only 44 (91.6%) were mannitol fermenter. 81 (93.1%) and 16 (18.3%) strains among 87 coagulase negative isolates were lactose and mannitol fermenters respectively.

Other biochemical reactions:

The strains showing positive M.R. and V.P. reactions were 94 (69.6%) and 69 (51.1%) respectively among total isolates. 45 (93.3%) and 37 (77%) strains were positive to M.R. and V.P. tests respectively in the coagulase positive group, while in the coagulase negative group 49 (56.3%) strains were positive for M.R. reaction and 32 (36.7%) possessed the capacity

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of acetyl methyl carbinol production (table IV).

The gelatin was liquefied by 45 (33.3%) of the total strains. 25 percent of the coagulase positive strains were found gelatin liquefier and 69.7 percent strains had capacity to elaborate gelatinase though coagulase negative.

Table IV further indicates that all the coagulase positive strains reduced nitrate into nitrite and had good growth in nutrient broth medium containing 10 percent sodium chloride, being salt tolerant strains. Among the 87 coagulase negative strains, 77 (85.5%) and 82 (94.2%) were positive for nitrate reduction and salt tolerance tests respectively.

Studies on pigment production by the strains employed in the present investigation as furnished in the table-IV indicates that golden and white coloured strains are equally distributed among the coagulase positive as well as coagulase negative strains. The absence of yellow colour among the coagulase positive group is conspicuous. It is, therefore, apparent that pigment production vary independently. The absence of yellow colour among the coagulase positive strains has also been reported by Cowan (1938). Smith (1947) failed to find any yellow coloured strain among the 110 pathogenic strains, while among the 63 nonpathogenic strains only two strains produced yellow pigment. Hence, the low incidence and negative coagulase activity of yellow coloured strains as reported in the present study is

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in full agreement with the finding of these workers. In absence of any report about the yellow coloured coagulase positive strains, it may be inferred safely that yellow coloured strains are coagulase negative or a short of inverse correlation exists between coagulase activity and yellow pigment production.

Inconsistant relation between coagulase activity and pigment production as observed in the present study, has also been reported by various previous workers including Slanetz & Bartley (1953), Reid & Wilson (1959) and George et al. (1962). Barber (1955) observed that change in pigment production could be made on subculturing. Seldom demonstration of aureus colour in some of the nonpathogenic staphylococci obtained from marine flora and associated with spoilage was reported by Wood (1952).

The present observation alongwith the conflicting reports on chromogenicity provides much justification to abandon this property as a criterion of classification.

The result on fermentation of lactose and mannitol revealed that lactose was fermented by majority of the coagulase negative strains and by all the coagulase positive strains. Mannitol was attacked by 91.6 percent of the coagulase positive strains but 18.3 percent of the coagulase negative strains also attacked this sugar with the formation of acid.

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Thus, the present study has indicated the high percentage of mannitol fermentors among coagulase positive strains. Inspite of the observation of Dudgeon (1908), Cruickshank (1937), Chapman and Stiles (1948) that mannitol fermentation, be regarded of differential value between pathogenic and nonpathogenic strains, the finding of the present study has supported the contention of Smith (1947), Saxena (1954) and Malik (1959). They regarded this property less reliable, as a criterion of differentiation because of the variable reactions, observed in their studies with pathogenic and nonpathogenic strains.

In the present study 45 strains out of 135 were found possessing the capacity to elaborate gelatinase. Only 25 percent of the coagulase positive strains while 69.7 percent of the coagulase negative strains liquefied gelatin. The property of liquefying gelatin had been regarded as characteristic of pathogenic strains as Smith (1947) observed almost complete correlation between gelatin liquefaction and coagulase production in staphylococci isolated from various animals. Edwards & Hippon (1957) found strains associated with severe mastitis and having marked virulence for mice produced rapid liquefaction of gelatin. Reid and Wilson (1959) also reported high percentage (93%) of gelatinase producing strains among those isolated from acute bovine mastitis. But Evans & Niven (1950) regarded this criteria as unsound for classification, as only 50 percent of either enterotoxin-positive or negative strains examined by them

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liquefied gelatin. Similarly Elek & Levy (1950) reported that 63 percent strains were gelatin liquefier among the 195 coagulase positive cultures. George et al.(1962) also regarded this property not a useful characteristic in distinguishing strains of staphylococci from milk. The present study has supported the view of workers who attached little differential value to this property. The lower percentage of gelatin liquefier found in this study than those reported by others may be attributed to different techniques used by the other investigators as well as the source of materials for isolation of the strains.

Perusal of Table-IV showing the results of other biochemical reactions of staphylococci has led to agree with the prevailing notion among research workers that these reactions may not be regarded as a dependable tool for the classification of staphylococci.

The result of salt tolerance by the strains of staphylococci as shown in table IV indicates that all except a few strains were salt tolerant. It was reported by Kock(1942) that growth of Staph.aureus was not inhibited by the addition of 7.5 percent sodium chloride to solid media and later confirmed by Chapman (1945). Kock (loc.cit.) even cultivated staphylococci in a liquid medium containing 15 percent sodium chloride. Parfentjev and Catalli (1963) though agreed with the view of above workers about the tolerance of high concentration of sodium chloride by staphylococci but reported discrepancy

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about 0.85 percent solution. They observed the injurious effect of the later concentration on the organism.

The present study has supplemented the claim of numerous workers that tolerance to sodium chloride in broth medium can be a helpful tool in differential diagnosis of this organism (Kock, loc.cit.; Chapman, loc.cit.; Smith, 1958 and Perfentjev & Catalli, loc.cit.).

Haemolysis:

The study on haemolysin production by the isolates was carried out by observing the lytic activity on the red cells of different species of animals. The results have been tabulated in table-V, VI and VII.

TABLE- V

	No.of strains	No.of haemolytic strains	Percentage
Total	135	44	32.5
Co.(+)	48	28	58.3
Co.(-)	87	16	18.3

Co.= coagulase

From the above table it appears that 58.3 percent of the coagulase positive strains were haemolytic

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and 18.3 percent strains produced haemolysin, though were coagulase negative .

The haemolytic strains were further examined for the production of different haemolysins viz., alpha, beta and delta, either alone or in combination. The number of strains producing different lysins alongwith percentage have been shown in table VI.

TABLE-VI

No. of strains		Haemolysin produced by strains of					
		Alpha		Beta		Delta	
		No.	%	No.	%	No.	%
Total	43	19	44.1	41	95.3	19	44.1
Co.(+)	27	12	44.4	26	96.2	13	48.1
Co.(-)	16	7	43.7	15	93.7	6	37.5

Co.(+) = Coagulase positive

Co.(-) = Coagulase negative.

Table VI reveals that beta haemolysin was produced by majority of the strains. 95.3 percent of the total haemolytic strains produced beta lysin while each alpha and delta lysin was produced by 44.1 percent isolates.

Strains showing different patterns of

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haemolysis as observed by plate method has been depicted in table-VII(overleaf).

Table-VII leads to infer that alpha haemolysin alone or in combination with delta lysin was not produced by any of the isolates. Beta haemolysin alone was produced by 6 strains each of coagulase negative and positive groups. 27.9 and 23.2 percent haemolytic strains produced alpha-beta and beta-delta lysins respectively. Only 4.7 percent of the haemolytic strains produced delta lysin alone, and 16.2 percent produced combination of alpha, beta and delta lysins.

To study the haemolysin production in the present study plate method has been adopted.This method has been regarded more sensitive than tube method for detection of individual haemolysins, owing to their differential diffusion rates. The chance of masking of reactions due to interaction of different haemolysins as apprehended in the tube test is abolished when plate method is adopted,(Elek & Levy,1954). The difficulty in distinguishing between alpha and beta lysins has been experienced as apprehended by other workers including Elek & Levy (1950), Blair (1958) and Malik (1959). Hence, the production of alpha, beta, and delta lysins by different strains has been confirmed by the use of anti-alpha haemolysin for Elek & Levy (loc.cit.) plates to avoid the visual error usually encountered with plate test.

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TABLE-VII

Different patterns of haemolysis shown by isolated strains of staphylococci.

No.of strains (Haemolytic)	Haemolytic patterns													
	Alpha		Beta		Delta		Alpha-beta		Alpha-delta		Beta-delta		Alpha-beta-delta	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Total 43	-	-	12	27.9	2	4.7	12	27.9	-	-	10	23.2	7	16.2
Co. + 27	-	-	6	22.0	1	3.7	8	29.6	-	-	8	29.6	4	14.8
Co. - 16	-	-	6	37.5	1	6.2	4	25.0	-	-	2	12.5	3	18.7

Co.+ = Coagulase positive

Co.- = Coagulase negative

During the present study, the strains of staphylococci from udder has been found to produce beta toxin to the extent of 95.3 percent irrespective of coagulase status, either alone or in combiantion with other haemolysins. Table-VI also reveals the extent of production of alpha and delta haemolysins by a good number of strains. As such, the present study supports the view held by Minett (1936), Smith (1947), Nakagawa (1958) and George et al.(1962) that beta toxin be regarded as characteristic of animal strains. The present finding tallies with the contention of Elek (1959) that 'in general the finding of a beta lysin producing strain in routine material is about ten times more likely if the strain originated from animal sources'.

Perusal of table-VII indicates that none of the isolates has produced alpha lysin, alone, while only two pure delta lysin producing strains have been found among the 43 haemolytic strains. The other patterns of haemolysin were beta, alpha & beta, beta & delta and alpha,beta and delta. Reid & Wilson (1959) reported the occurrence of two strains which produced only delta lysin among their 28 cultures from chronic mastitis but none of them was pure alpha lysin producer. The rest of their cultures produced combination of alpha, beta and delta lysins. The report on alpha haemolysin alone producing strains of Slanetz & Bartley (1953) and Edwards & Rippon (1957) may be assumed as the characteristic of the particular strains examined by them.

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The conflicting report along with the present one, about the pattern of combination has failed to suggest any definite pattern of haemolysis which might be considered as characteristic of animal strain.

In the present study 58.3 percent of the coagulase positive strains have been found haemolytic while 18.83 percent of coagulase negative strains are also haemolytic. The percentage of haemolytic strains among the coagulase positive isolates has been found lower than the findings of Williams and Harper (1947) who reported agreement between these two characters upto 90 percent. Jackson (1955) and his colleagues found it only to the extent of 82 percent. Joshi and Dale (1963) found 93 percent of their 154 coagulase positive strains to be haemolytic, though Cowan (1938) and Christie et al.(1946) observed complete correlation between these two characters. The observation of Daranyi (1926), Keogh (1940) and Joshi & Dale (loc.cit.) about the occurrence of coagulase negative haemolytic strains,supports the findings of the present investigation about coagulase negative yet haemolytic strains.

Keeping in view, the above observations it may be inferred that inspite of variable correlation between coagulase activity and haemolysin production; haemolytic behaviour of staphylococci does give an insight to the role of this property vis-a-vis pathogenicity.

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Coagulase activity:

The coagulase activity of 135 strains was studied by employing slide technique and tube technique separately. Rabbit plasma was used in slide technique and rabbit and human plasma were employed separately in tube technique for the examination of coagulase activity of isolated strains. The results of both the techniques appear in the table-VIII.

TABLE - VIII

No. of strains	No. of strains showing positive result		
	Tube technique		Slide method
	Human plasma	Rabbit plasma	C rabbit plasma
135	48	48	44

From the above table it appears that identical results were obtained in detecting the coagulase positive strains by tube method employing either rabbit or human plasma. The slide test was not successful for the detection of only four strains which were found coagulase positive by tube method. Thus, the agreement between slide and tube methods came to 91.6 percent considering the result

of the tube technique to be 100 percent.

The suitability of rabbit and human plasma in detection of coagulase positive strains of staphylococci has been advocated by Chapman et al.(1938), Klatt (1954), Nakagawa (1958) and numerous investigators. In the present study also the plasmas of rabbit and human have been employed separately for the detection of coagulase activity in isolated strains. Perusal of table-VIII indicates that rabbit and human plasma have behaved in a similar manner in the detection of coagulase positive strains. Thus, it establishes the fact about the suitability of either plasma in judging the coagulase activity of staphylococci, as observed by above workers.

The agreement between the results of tube method originally used by Loeb (1903) and recently devised slide technique of Cadness-Graves et al.(1943) has been found to the extent of 91 percent. This result is slightly better than those reported by Penfold (1944) and Malik (1959). However, Smith (1947) has reported identical results with these two methods when used separately.

The fluctuation in the results between the slide and tube methods can be explained with the help of the view expressed by Duthei (1954) about the existence of two forms of staphylococcal coagulase. He brought forth the evidence of 'bound coagulase' attached to the cell surface responsible for slide clumping and 'free coagulase' which would liberate in the medium

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during growth being responsible for the tube coagulase test. The other factors which may be attributed as the possible reasons of variation in the report about the results of tube and slide technique may be the type of animal plasma used - a highly variable material and the amount of coagulase production which may vary from strain to strain.

With the above observations, it may be concluded that either human or rabbit plasma could be employed in detection of coagulase positive strains of staphylococci. The slide method can be used as a useful screening test for the presumptive recognition of coagulase positive strains. Inspite of the objection raised by Lack & Wailling (1954), the tube test may be regarded as superior to slide test in detecting the coagulase positive strains, though the former test needs more care and time in carrying out the same.

<u>Correlation of important in vitro properties of staphylococci in milk from apparently healthy bovine udders:</u>

The correlation between the important physiological characters of 135 strains isolated from milk of apparently healthy bovine udders have been depicted in table-IX, X and XI.

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TABLE-IX

Correlation of important *in vitro* properties of staphylococci.
No.of strains 135.

Particulars		Coagulase activity.		Colour			Mannitol ferment.		Haemolysin production		M.R.		V.P.		Gelatin liquefaction	
		+	-	G	W	Y	+	-	+	-	+	-	+	-	+	-
No.of strains		48	87	62	69	4	60	75	44	91	94	41	69	66	45	90
Coagulase	+			21	27	-	44	4	28	20	45	3	37	11	12	36
	-			41	42	4	16	71	16	71	49	38	32	55	33	54
Colour	G						36	26	31	31	48	14	37	25	23	39
	W						24	45	13	56	45	24	32	37	21	48
	Y						-	4	-	4	1	3	-	4	1	3
Mannitol fermentation	+								38	22	54	6	45	15	21	39
	-								6	69	40	35	24	51	24	51
Haemolysin production	+										42	2	38	6	13	31
	-										52	39	31	60	32	59
M. R.	+												64	30	30	64
	-												5	36	15	26
V. P.	+														23	46
	-														22	44

G= golden yellow; W= white; Y= yellow.

TABLE - X

Correlation of important in vitro properties of staphylococci. No. of strains 135.

Particulars	Coagulase activity.		Colour			Mannitol fermentation		Haemolysin production		M.R.		V.P.		Gelatin liquefaction	
	+	-	G	W	Y	+	-	+	-	+	-	+	-	+	-
No. of strains	48	87	62	69	4	60	75	44	91	94	41	69	66	45	90
Coagulase +			33.8	39.1	-	73.3	5.3	63.6	21.9	47.8	7.3	53.6	16.6	26.6	40.0
Coagulase -			66.1	60.8	100	26.6	94.6	36.3	78.0	52.1	92.6	46.3	83.3	73.3	60.0
Colour G						60.0	34.6	70.4	34.4	51.0	34.0	53.6	37.8	51.1	43.3
Colour W						40.0	60.0	29.5	61.5	47.8	58.5	46.3	56.0	46.6	53.3
Colour Y						-	5.3	-	4.3	1.0	7.3	-	6.0	2.2	3.3
Mannitol fermentation +								86.3	24.1	57.4	14.6	65.2	22.7	46.6	43.3
Mannitol fermentation -								13.6	75.8	42.5	85.3	34.7	77.2	53.3	56.6
Haemolysin production +										44.6	4.8	55.0	9.0	28.8	34.4
Haemolysin production -										55.3	95.1	44.9	90.9	71.1	65.9
M. R. +												92.7	45.5	66.6	71.1
M. R. -												7.2	54.5	33.3	28.8
V. P. +														51.1	51.1
V. P. -														48.8	48.8

G = golden yellow; W = white; Y= yellow.

Distribution of properties depicted along the vertical line within

(in %) the group (no.of strains) having property as indicated along the horizontal line.

TABLE- XI

Correlation of important *in vitro* properties of staphylococci. No. of strains 135.

Particulars		No. of strains.	Colour			Mannitol fermentation		Haemolysin production		M. R.		V.P.		Gelatin liquefaction	
			G	W	Y	+	-	+	-	+	-	+	-	+	-
Total isolates		135	45.9	51.1	2.9	44.4	55.5	32.5	67.4	69.6	30.3	51.1	48.8	33.3	66.6
Coagulase	+	48	43.7	56.2	-	91.6	8.3	58.3	41.6	93.3	6.3	77.0	22.9	25.0	75.0
	-	87	47.1	48.2	4.5	18.3	81.6	18.3	81.6	56.3	43.6	36.7	63.2	37.9	62.0
Colour	G	62				58.0	41.9	50.0	50.0	77.4	22.5	59.6	40.3	37.0	62.9
	W	69				34.7	65.2	18.8	81.1	65.2	34.7	46.3	53.4	30.4	69.4
	Y	4				-	100	-	100	25.0	75.0	-	100	25.0	75.0
Mannitol fermentation	+	60						63.3	36.6	90.0	10.0	75.0	25.0	35.0	65.0
	-	75						8.0	92.0	53.3	46.6	32.0	68.0	32.0	68.0
Haemolysin production	+	44								95.4	4.5	86.3	13.6	29.5	70.4
	-	91								57.1	42.8	34.0	65.9	35.1	64.8
M. R.	+	94										68.0	31.9	31.9	68.0
	-	41										12.1	87.7	36.5	63.4
V. P.	+	69												33.3	66.6
	-	66												33.3	66.6

G = golden yellow; W = white; Y= yellow.

Distribution of properties depicted along the horizontal line within (in %) the group (no.of strains) having properties as indicated along the verticle line.

Perusal of table-IX & X indicates that of the total 135 strains examined, only 48 were found coagulase positive and the rest 87 strains were coagulase negative. On the basis of chromogenicity 62 strains were golden yellow in colour while 69 were white and only 4 being yellow. Overall picture of the table IX, X & XI indicates lack of complete correlation between golden yellow colour and coagulase activity of the isolated strains as neither all the golden yellow coloured strains were coagulase positive nor the reverse was absolute. The coagulase positive strains were made up of 21 (43.7%) golden yellow and 27 (56.2%) white coloured strains. None of the yellow coloured strains was included in coagulase positive group. The golden coloured strains also found place among the coagulase negative group.

The correlation between coagulase activity and haemolysin production was also found incomplete as only 28 (58.3%) strains were haemolytic among the 48 coagulase positive strains,& 16 (18.3%) strains were haemolytic in coagulase negative group. Reversely of the 44 haemolytic strains 63.6 percent were only coagulase positive. It can also be inferred from these tables that out of 44 haemolytic strains 31 were golden yellow and 13 white. Yellow coloured strain could not find place among the haemolytic strains.

The other biochemical tests such as mannitol fermentation, gelatin liquefaction, M.R. and V.P.

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reactions could not be correlated with either coagulase activity or haemolysin production. Gelatin was liquefied by only 45 strains out of 135 isolates i.e. 23 out of 62 golden coloured strain; 12 out of 48 coagulase positive strains and 13 out of 44 haemolytic strains and likewise other tests also gave variable results and no definite correlation could be observed.

Antibiotic sensitivity of isolated strains:

The sensitivity of all the 135 strains of staphylococci isolated from milk of the apparently healthy bovine udders were carried out against three commonly used antibiotics viz., Penicillin, Streptomycin and Terramycin. The growth of majority of strains was observed to be significantly affected in the presence of some antibiotics incorporated in the medium, while some were found remarkably resistant. Some species were found showing the sensitivity or resistance beyond the dilution used. Among the antibiotics employed in this investigation streptomycin and terramycin were found inhibiting the growth of all strains of staphylococci in significant dilutions while, penicillin was observed ineffective on some of the strains even at much higher dilution.

The number of strains found sensitive or resistant at various concentration of penicillin incorporated in the medium has been depicted in table-XII.

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TABLE-XII

Results of penicillin sensitivity test of the isolated strains of staphylococci.

No. of strains	Concentration of penicillin per ml. in the basal medium																	
	0.05 I.U.		0.10 I.U.		0.25 I.U.		0.5 I.U.		1.0 I.U.		1.5 I.U.		150 I.U.		1500 I.U.		1500 I.U.	
	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R
135	91	44	99	36	114	21	120	15	121	14	123	12	126	9	130	5	133	2

S = Sensitive ; R = Resistant

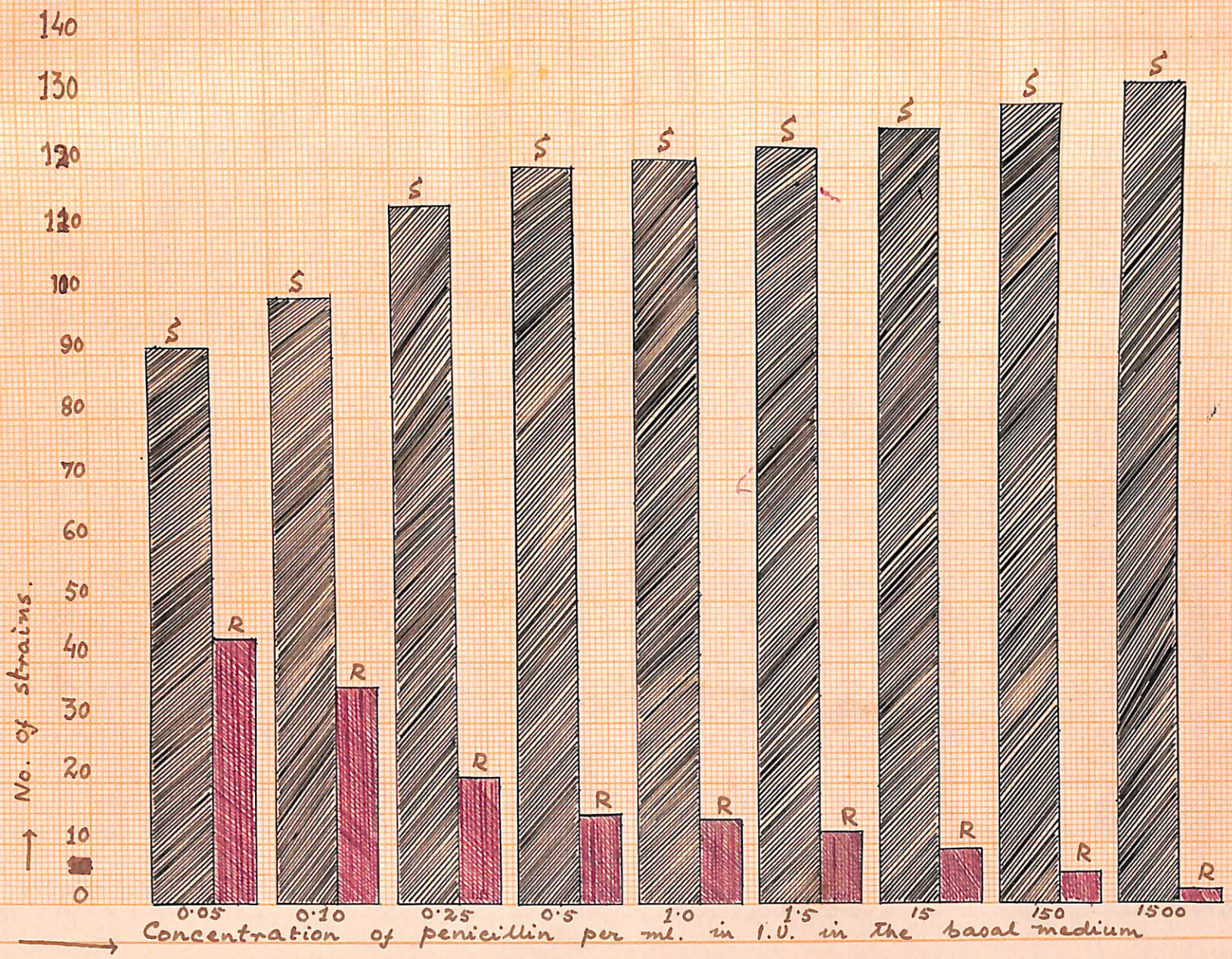
TABLE-XIII

Important physiological characters of penicillin sensitive and resistant strains

No. of strains.		Colour			Coagulase		Haemolysin		Mannitol fermenter.		Gelatin liquefaction	
		Golden	White	Yellow	+	-	+	-	+	-	+	-
Penicillin sensitive	120	53	63	4	44	76	41	79	55	65	39	81
Penicillin resistant	15	9	6	-	4	11	3	12	5	10	6	9

S- Sensitive
R- Resistant

HISTOGRAM SHOWING NO. OF RESISTANT & SENSITIVE SRAINS AT DIFFERENT CONCENTRATIONS OF PENICILLIN IN THE BASAL MEDIUM.



Perusal of table-XII indicates that majority of the strains were quite sensitive at the initial concentration of 0.05 I.U. per ml. of basal medium. The number of strains found sensitive at 0.05, 0.1, 0.25 and 0.5 I.U. per ml. of concentration in the basal medium were 91, 99, 114 and 120 respectively. The increase in the sensitive strains beyond this concentration was found 1, 2, 3, 4 and 3 respectively, though the concentration of penicillin was increased to 1.0, 1.5, 15, 150 and 1500 I.U. per ml. of the basal medium. The growth of two strains were not inhibited even at an exorbitantly high concentration of 1500 I.U. per ml. of the basal medium.

The details of the physiological characters of penicillin resistant strains,(0.5 I.U./ml.concentration) has been furnished in table XIV and the important ones shared by sensitive as well as resistant strains has been depicted in table XIII.

Table XIII indicates only 4 out of 15 strains produced coagulase while haemolysin production was observed in 3 only. None of the yellow coloured strain found place among the resistant strains. Mannitol was fermented by 5 resistant strains. Overall picture of the physiological characters depicted in table XIV of resistant strains suggested that they were not physiologically active.

Staphylococcal sensitivity to commonly used antibiotics in the present study gives an encouraging

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TABLE- XIV

Physiological characters of penicillin resistant staphylococci isolated from milk of apparently healthy udders.

Sl. No.	Culture No.	Colour	Mannitol fermentation.	Coagulase activity.	Gelatin liquefaction	Haemolysin production	M.R.	V.P.
1.	18	W	-	-	+	-	+	+
2.	25	W	-	-	-	-	-	-
3.	27	G	+	+	-	-	+	-
4.	28	W	+	+	+	-	-	-
5.	48	G	-	-	+	-	-	+
6.	52	W	-	-	-	+	+	-
7.	67	G	+	-	+	+	+	+
8.	71	G	-	-	-	-	-	-
9.	73	G	-	-	-	-	-	-
10.	84	G	-	-	-	-	-	-
11.	90	G	-	-	-	-	-	-
12.	106	G	+	+	-	+	+	+
13.	127	W	+	+	-	-	+	+
14.	136	G	-	-	+	-	-	+
15.	146	W	-	-	+	-	-	-

G = Golden yellow

W = White

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picture in the herd in question. All the 135 isolates have been found sensitive to Streptomycin and Terramycin. Penicillin has been found to have no inhibitory effect on 15 strains out of 135 at 0.5 I.U. per ml. concentration in the basal medium.

Although Penicillin and other antibiotics are being used extensively in the treatment of animal diseases including udder infections, the present evidence is that this has failed to result in a build up of antibiotic resistant staphylococci in the udder.

Seto & Wilson (1954) reported only 12 percent cultures of staphylococci were resistant to penicillin out of 196, isolated from milk. In addition, they also found 3 strains being resistant to streptomycin and one to chlortetracycline and oxytetracycline. Schalm and Woods (1953) found variation in the number of resistant strains between the herd of two farms. Malik (1959) and George et al.(1962) also demonstrated the percentage of penicillin resistant strains to be low in udder. Zinn et al.(1961) reported only 12 resistant strains among the 203 cultures isolated from milk. But Panduranga Rao et al.(1966) found 85.5 percent of the strains being sensitive to penicillin and 86.5 percent to streptomycin out of 137 strains of Staph. aureus but the isolation was from cases of bovine mastitis. The role of milk in the dissemination of antibiotic resistant staphylococci was suggested by many, but the present investigation along with the report of above workers does not support the

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contention that milk from apparently healthy bovine udder is an abundant source of antibiotic resistant staphylococci for man.

Perusal of table-XII indicates that the number of sensitive strains swelled with the increasing concentration of antibiotics (Penicillin) in the basal medium. The increase in number upto the level of 0.5 I.U. per ml. concentration is apparent, above which only a little effect of increasing concentration is seen on the increase of sensitive strains. Malik (loc.cit.) observed inhibition of all his strains of staphylococci including the positive control at 100 I.U./ml. concentration but in contrast two strains of the present study have been found unaffected even at an exorbitantly high concentration of 1500 I.U. per ml. of penicillin in the basal medium. Sinha (1961) reported the occurrence of such strains of staphylococci found resistant at 1500 units of Penicillin isolated from human beings.

Thus, the present study shows that penicillin still holds its position in its effect on majority of the staphylococci from udder. The observation that the decrease in the number of resistant strains was not appreciable beyond 0.5 I.U. per ml. concentration of penicillin in the basal medium leads to conclude that even higher doses of penicillin to combat the staphylococcal infection due to resistant strains is not worth consideration.

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In the present study, serial dilution method has been preferred to examine the susceptibility of strains towards antibiotics for the direct and straight forward sensitivity result,it gives. Also, in practice, dilution techniques have been regarded as of particular value in determining whether an infection may logically be treated with high doses of an antibiotic to which the pathogen would normally be considered resistant by qualitative criteria. The dilution method could be employed either in liquid medium or solid medium. The later has been chosen in the present study keeping in mind the better working facility, several strains could be streaked on a single plate and plates once prepared could be preserved in refrigerator for future use without any appreciable loss of the potency of penicillin incorporated in the medium for a week (Erlarson,1951). In addition, contamination if any, could be spotted easily and chances of error in observing the result comes to minimum than the tube dilution method in which the visual error in interpretation of result comes infront. This method has been successfully employed by several other workers(Erlarson,loc.cit.; Wallmark, 1954 and Malik,loc.cit.).

Various factors, like dose of inoculum and time of incubation has been reported affecting the judgement in selection of sensitive or resistant strains by many workers (Luria, 1946; Spink, 1951 and Diding & Wallmark,1951). However, attempts have been made to carry out the sensitivity test of the isolated staphylococci during the present study under standard condition.

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Only those strains have been treated resistant in the present investigation which grew at 0.5 I.U. per ml. concentration of penicillin in the basal medium as adopted by Malik (loc.cit.) in examining the sensitivity of staphylococci. Uptil now no uniform opinion has been formed regarding the fixation of limit of penicillin in the basal medium while keeping all other factors standard. This aspect of the problem of the drug sensitivity is still engaging the attention of several workers. While, Per Oeding (1952) used 0.08 I.U. or higher per ml. concentration of penicillin for the inhibition of growth in resistant strains, Chaub & Foley (1952) considered 0.6 unit per ml. concentration as limit. Erlarson (loc.cit.) regarded more than 2 I.U. per ml. concentration for the inhibition of growth in resistant strains.

Perusal of above report suggests that there is lack of uniformity in the opinion amongst the workers about the limit of penicillin concentration in the basal medium. In the present study, the limit of 0.5 I.U. per ml. has been considered reasonable keeping in view the fair margin which it maintains with the therapeutic level of penicillin in blood.

The other aspect of the penicillin sensitivity which merit discussion, is the relation of important physiological characters with the development of resistance in organism.

Table-XIII & XIV disclose that majority of resistant strains were found negative for most of the in vitro

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tests employed to examine the physiological properties of staphylococci. The question crops up, has resistance a say in the disappearance of the properties including coagulase activity and haemolysin production ? If it is so, the inference can be drawn that the circumstances which led to the development of defensive mechanism in staphylococci against antibiotics also brought about other changes in their physiological properties considered so vital an index of pathogenicity. A review of the available literature provides a conflicting report on this aspect of drug resistance.

McVeigh & Hobdey (1952) and Massieu (1960) have reported about antibiotic resistant strains, which lost the property of coagulase production. But Barber (1953) reported about penicillin tolerant strains of staphylococci. These strains could be isolated with great frequency when penicillin sensitive staphylococci were passaged in the presence of penicillin *in vitro* according to him. They were reported to be deficient in coagulase and alphatoxin production. But penicillin destructive strains which he considered the cause of penicillin resistant staphylococcal infection, resembled penicillin sensitive strains in all respect except the capacity to produce an enzyme penicillinase which inactivates penicillin. This enzyme is present in resistant strains but absent in the sensitive one.

Uniformity in views on the mode of origin of penicillinase producing staphylococci is also lacking. Though

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most of the workers regarded enzyme adaptation as possible mode of origin, others speak of 'selection! Yet another group of worker consider spontaneous mutation as the way of origin of this group of organisms.

Barber (1953) reported the isolation of staphylococci having weak penicillin destroying activity from two penicillin sensitive coagulase positive strains of staphylococci on prolonged exposure to penicillin. He observed diminution in growth rate, coagulase and alphatoxin production and having lower order of resistance in these isolates than the laboratory strains. He regarded loss in properties usually associated with pathogenicity as merely a laboratory phenomenon and presumed that this would not occur in vivo.

In contrast to the above prediction about the in vivo produced penicillin destroying strains, the observation of the present study is suggestive of the another way. In the present study majority of penicillin resistant strains have been found physiologically not active. It is possible that these organisms lost their properties while acquiring resistance against penicillin. Thus, the loss in physiological characters in in vitro produced strains as observed by the above workers may be applicable to in vivo originated strains too. As the penicillinase status of the strains have not been examined, it may be taken as guess based on the informations available.

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Pathogenicity test of strains on mice:

Sixty four strains out of 135 isolates were tested for the pathogenicity in adult Swiss albino mice. The strains were selected on the basis of *in vitro* tests for coagulase activity and haemolysin production. While sorting out the strains, resistance of the isolates to penicillin was also kept in mind.

Out of 64 strains, 33 were regarded pathogenic and 31 nonpathogenic. Death of 29 mice among the total of 128 was observed within seven days, while organisms from the rest mice were recovered on blood agar plate when they were sacrificed on the eighth day. Only 37 mice were found infected as the organisms were recovered from the heart blood of these mice only. The necropsy of the mice which died during the period of observation revealed marked congestion and enlargement of spleen. Engorgement of blood vessels in the peritonium was also evident. The organisms were recovered in pure culture from the heart blood.

The type of organisms based on the *in vitro* properties and other relevant information have been depicted in table-XV.

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TABLE-XV

Result of pathogenicity test on mice of staphylococci alongwith their characters.

Type of organisms.	No. of strains	No.of mice inoculated	No.of mice infected	Percentage of infection
CS	14	28	12	66.6
CHS	23	46	40	86.5
HS	13	26	4	15.3
CR	3	6	4	66.6
CHR	1	2	2	100.0
HR	2	4	-	-
R	8	16	4	25.0
Total:-	64	128	66	51.5

C = Coagulase positive

H = Haemolysin positive

S = Sensitive to penicillin

R = Resistant to penicillin

From table-XV above, it appears that out of 128 mice inoculated with 64 test strains of staphylococci only 66 picked up infection which formed 51.5 percent of total mice inoculated. 86.5 percent of the mice were infected with the strains possessing properties of coagulase and haemolysin production.

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Of the coagulase negative haemolytic strains, the percentage of infection in mice was found 15.3 percent and coagulase positive nonhaemolytic strains produced infection in mice to the extent of 66.6 percent.

Similarly, among the penicillin resistant strains none of the two coagulase negative isolates proved infective. The only strain positive for haemolysin and coagulase production produced infection in both the mice and three coagulase positive nonhaemolytic strain, each inoculated in two mice produced infection in 4. Among this group 16 mice inoculated with eight penicillin resistant strains devoid of coagulase activity and haemolysin production, four mice were found to have picked up infection.

Distribution of important *in vitro* physiological characters in pathogenic and nonpathogenic strains based on mice pathogenicity test has been depicted in table-XVI(overleaf). It shows that out of 33 pathogenic strains, 28 produced coagulase; 23 were golden yellow in colour and rest were white coloured; 31 fermented mannitol, 23 were haemolytic and 30 & 31 strains were positive for M.R. and V.P. reactions respectively, but gelatin was liquefied by eight strains only and five were resistant to penicillin. Thus, it gives fair idea about the contention of various workers who utilised single *in vitro* property of staphylococci to recognise the pathogenic strains of this group of organisms.

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TABLE-XVI

Distribution of important *in vitro* physiological properties among pathogenic and nonpathogenic staphylococci.

	No. of strains	No. of strains showing positive results for									
		Colour		Coagulase.	Haemolysin.	Mannitol fermentation	M.R.	V.P.	Gelatin liqu.	Penicillin sensitivity	
		G	W							R	S
Pathogenic	33	23	10	28	23	31	31	30	8	5	28
Nonpathogenic.	31	15	16	13	17	20	23	19	15	9	22

G = Golden yellow

W = White

R = Resistant

S = Sensitive.

Perusal of table-XV indicates that the strains possessing both the properties i.e. coagulase and haemolysin production, have caused infection in maximum percentage (86.5%) of mice. The coagulase negative but haemolytic strains have affected least percentage (15.3%) of mice while coagulase positive non-haemolytic strains have occupied the intermediate position by infecting 66.6 % of mice. Among the penicillin resistant strains also, similar picture of infection in relation to physiological properties has been observed though two strains lacking in both these properties have proved infective.

Though, the very basis of this test in the evaluation of pathogenicity of strains is not free from doubts and other limitations, results of the test has proved helpful in deducing certain conclusions on the pathogenicity of test strains in relation to some physiological characters. Christie et al. (1946) and Pisu (1951) attached much importance to this test in judging the pathogenicity of organisms while Smith (1947) and Edwards & Rippon (1957) raised pointing finger about the suitability of this test. The animal pathogenicity tests as a whole was regarded not satisfactory by Barber (1947), as he felt that human and laboratory animals react differently to staphylococcal infection. Also, different routes alongwith arbitrarily fixed does of culture or number of organisms forming the inoculum were used in mice to test the pathogenicity

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of strains by various workers. Minett (1936) used 0.1 cc. of the 24 hrs. broth culture as inoculum to test the pathogenicity of staphylococci in mice whereas 0.2 cc. of 18 hrs. broth culture and intramuscular route was adopted by Selbie & Simon (1952). Ekstedt & Yotis (1960) preferred intracranial route for the demonstration of role of coagulase in increasing the virulence of haemolytic strains but Schneierson et al.(1961) employed intraperitoneal route.

In the present study 0.4 cc. of 18 hrs. broth culture has formed the inoculum and intraperitoneal route has been employed as used by Natarajan (1964) in his study on Staph. aureus of bovine udder origin.

The results of the present investigation have provided many corolaries. The first being that either the production of haemolysin or coagulase alone may not be taken as an indication of pathogenicity with certainty whereas positive for both is strong indication as inferred on the basis of the result of the present investigation.

The coagulase activity of staphylococci has been regarded by numerous workers as an index of pathogenic strains. Since Daranyi (1926) reported the association of this character with pathogenicity, Cowan (1938), Smith (1947) and Smith & Dubos (1956) investigated and found close correlation between coagulase activity and virulence in this group of organisms. The demonstration of Eksfedt & Yotis (loc.cit.) on

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mice that partially purified coagulase increased the virulence of haemolytic, noncoagulase producing strain of staphylococci gave strength to the contention of above workers.

The other group of workers considered production of haemolysin to be associated with virulence after their studies on mice,(Christie et al.,1946; Selbie & Simon,1952 and Anderson,1956).

But, during the present study coagulase activity alone has not proved to be a strong indication of virulence. The findings about the haemolysin production in relation to the pathogenicity of strains have been found discouraging. The discrepancy in the result of the present investigation with those of the past workers may be due to differences in experimental procedure and the particular batch of strains examined by them. The view of Barber (1960) that a positive coagulase test be considered simply a guide to detect the potential pathogen which does not necessarily imply full pathogenicity and gives no indication of virulence may be regarded as support of the present finding.

The contention about the haemolytic noncoagulase positive strains agrees with those of Schalm and Woods (1953), Slanetz & Bartley (1953) and Nakagawa (1958) who demonstrated the occurrence of coagulase negative haemolytic strains of staphylococci in bovine udder and considered them as nonpathogenic. The finding tallies with the observation of

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Dolman (1932) and Tager and Hales (1947), that in actual practice no correlation could be demonstrated between alpha lysin production and severity of the condition.

The result of the present study suggests that coagulase activity and haemolysin production, both have complementary role to speak of pathogenicity of a strain than either alone. It seems that Ekstedt & Yotis (loc.cit.) who praised the role of coagulase in increasing the virulence, designed their experiment to demonstrate the complementary role of this property with haemolysin production by choosing haemolytic strains only. Replacement of haemolytic strains used by them with coagulase negative, nonhaemolytic strains would have better explained the role of coagulase. The idea has also been appreciated by the workers themselves. The idea held by Lack & Wailling (1954) that no single property is complete enough to be used as criterion of pathogenicity, rather the wide spectra of toxin be regarded as an indication of pathogenicity, gets supported by the present finding. The conclusion of Burns and Holtman (1960) that staphylococcal pathogenicity is the result of various factors and virulent staphylococci which forms a group though 'physiologically active' and 'biochemically consistent' cannot be identified by any single morphological or biochemical criteria is endorsed by the present study.

The results of present investigation share the view of Amsterdam & Schneierson (1957) that no significant correlation exists between the penicillin susceptibility

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and virulence, but disagrees with their findings of higher proportion of resistant strains being pathogenic, for only three out of 14 resistant strains were regarded as pathogenic during this study. This contention gets support on perusal of physiological activities of resistant strains.

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S U M M A R Y

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S U M M A R Y

A brief review of some past and recent studies concerning the physiological characters of staphylococci and its application in the classification of this organism into pathogenic and nonpathogenic strains has been cited. The literature on association of staphylococci in relation to bovine mastitis and human food poisoning due to consumption of milk and milk products from infected udders has also been reviewed.

In the present investigation attempts were made to enquire into the characters of staphylococci from milk samples of the apparently healthy udders of cows. The samples were taken with such a case that it minimized the possibility of contamination from extraneous sources.

Altogether 371 samples from 94 cows in milk were examined and 135 strains of staphylococci were isolated. On the basis of positive coagulase activity, 48 strains were regarded *Staphylococcus aureus* irrespective of pigment production. Considering the coagulase status as an index of pathogenicity, the percentage of cows suffering from subclinical udder infection was found 51 percent.

Studies on the physiological characters of 135 strains of staphylococci have shown that chromogenicity being a variable character can not be relied upon for the differentiation of pathogenic and nonpathogenic strains. The absence of yellow pigment producing strains among the coagulase positive group led to think about the nonpathogenicity associated with this pigment. Both golden yellow and white coloured strains found place in the coagulase positive as well as negative groups.

Majority of the strains, irrespective of coagulase activity fermented lactose. 91.6% of the coagulase positive strains attacked mannitol with the production of acid while a small percentage of coagulase negative strains (18.3%) were also mannitol fermenters.

Similarly, other biochemical reactions were found shared by both coagulase positive and negative strains. Thus, this basis was not judged suitable to differentiate between coagulase positive and negative strains.

Good growth of (96.3%) strains which included all the coagulase positive isolates in nutrient broth medium containing 10% sodium chloride, supplemented the idea held by various workers regarding this test for differential diagnosis of this group of organisms.

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The examination of the strains for haemolysin production revealed that 95.3 percent of the haemolytic strains were beta toxin producers either alone or in combination with other lysins. None of the strains produced alpha lysin alone.

Regarding the coagulase activity, rabbit and human plasma gave identical results in tube technique. The slide test was found a good guide in the identification of presumptive pathogen, ie. coagulase positive strains. The agreement in the results of tube test and slide test was found 91.7 percent.

Study of correlation between important *in vitro* physiological properties could not lead to any definite conclusion.

The isolates were examined for their sensitivity towards commonly used antibiotics. Streptomycin and terramycin could not locate any strain resistant to them which suggested about their therapeutic value. Only 11.1% strains were found resistant at 0.5 I.U. per ml. concentration of penicillin in the basal medium by serial plate dilution method. The resistant strains were further carried to higher concentration of penicillin. It was found that they retained this property upto higher concentration. The result of this

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suggested that penicillin still retained its therapeutic value against staphylococci of udder, it is not advisable to attempt to treat the infection due to resistant organisms by increasing the dose.

Physiological properties of resistant strains provided a picture as if majority of these strains have lost their important physiological properties. Pathogenicity test of these strains on mice gave an indication that majority of the strains were nonpathogenic. Only three out of 14 resistant strains included in this test suggested to have pathogenic properties.

The pathogenicity of strains having coagulase activity and haemolytic properties when tested on mice led to conclude that the presence of both the properties rather either of them alone, be regarded as an indicator of pathogenicity of a strain.

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