ABSTRACT OF THESIS

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Title of Thesis Studies on Actinobacillus equuli

The morphological, cultural and biochemical characteristics of 138 strains of Actinobacillus equuli from various sources have been investigated and evaluated in order to provide useful criteria for the identification of this taxonomically disputed species of microorganism.

The antigenic structure of all these strains was investigated by agglutination and agglutinin-absorption tests and 98.6 per cent. of them can be arranged into 28 groups on the basis of their heat-stable antigens (O antigens). The heat-labile antigens associated with extracellular slime have been demonstrated and found to be relatively common to organisms of different O groups. Although agglutination tests offer a means of classifying this serologically heterogeneous group of organisms, the autoagglutinability of rough strains and inagglutinability due to its viscous nature have often been found to interfere with such tests. Thus the feasibility of applying other serological procedures to overcome the difficulties has been sought and a passive haemagglutination test and an immunodiffusion precipitin test for serogrouping of A. equuli were developed. The conditions for the passive haemagglutination test have been investigated and are discussed in some detail with special reference to the soluble antigens of A. equuli. Heat extracts of the organisms were used in the immunodiffusion precipitin test to group A. equuli strains into 28 O-groups. The immunodiffusion precipitin test appeared to be the test of choice for the serogrouping of isolates of A. equuli because of its specificity and its simplicity and rapidity in performance. Moreover, the immunodiffusion precipitin test has a great advantage over the other serological methods in showing a clear proof of an antigenic relationship between O groups.

The work has shown that serological classification of the organism is fairly well correlated with certain biochemical characters of the organism and the majority of the strains can be arranged into two main divisions. The term biotype is applied for the differentiation

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of these divisions of A. equuli. Of the 138 strains studied, 84 were of Type I, 42 were of Type II and the remaining 12 were intermediate in type. Type I strains gave a positive reaction with mannitol, whilst their reaction with salicin and cellobiose were negative. All the strains comprising Type I were aesculin-negative and, with the exception of one, non-haemolytic. These strains fell into O groups 1 - 22 inclusive. On the other hand, Type II strains were mannitol negative, whilst the majority of the strains fermented salicin and cellobiose and hydrolysed aesculin, and all haemolysed sheep erythrocytes. The Type II strains were made up of O groups 24 - 28 inclusive.

An antigenic relationship between A. equuli, A. suis and A. lignieresi has been demonstrated. Comparative biochemical and serological studies of these 3 species of Actinobacillus have shown that A. suis is closely related to Type II of A. equuli and that A. suis differs sufficiently from both A. lignieresi and Type I of A. equuli to warrant its separation as a different species within the genus.

Further work is required to characterise haemolytic actinobacilli from horses and pigs, but the results obtained in the work appear to suggest the not infrequent occurrence in the upper respiratory tract of normal horses of organisms resembling A. suis and the association of such organisms with disease in this host species on occasions.

STUDIES ON

ACTINOBACILLUS EQUULI

BONG HWAN KIM

A thesis presented for the degree of

Doctor of Philosophy

of the University of Edinburgh

1976

TO

MY FAMILY

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FOREWORD

Actinobacillus equuli has been recognised since the beginning of this century as an important cause of neonatal death in foals in most countries of the world and considered to be responsible for as high a proportion as 25 per cent. of all infections in newborn foals. A. equuli infection in foals most often takes the form known as "sleeper" or "sleepy foal", an acute septicaemia of newborn foals which is one of the most common diseases to which foals are subjected to, at or shortly after birth. Moreover, evidence is accumulating that A. equuli and other closely related organisms produce a variety of clinical syndromes especially in piglets.

However, it is apparent from a review of literature that only slight attention has been given to the organism, so that even its taxonomic position is still uncertain. Some serological investigations on A. equuli have been carried out primarily to provide a basis for serological diagnosis and for serum therapy of the disease. Specific treatment of foals with an antiserum prepared from A. equuli or with dam's blood has been attempted but without encouraging results. Serological diagnosis and immunoprophylaxis of the disease have been considered to be impractical mainly because of the extreme heterogeneity of the organism. Nevertheless, the antigenic structure of the organism is virtually unknown.

Moreover, no satisfactory serological test is available for this purpose.

Haemolytic and non-haemolytic actinobacilli have been reported as a cause of acute generalised infection in pigs. The non-haemolytic actinobacilli have been identified as A. equuli, but there is much controversy over the taxonomic position of the haemolytic actinobacilli from pigs. As it is apparent that similar haemolytic organisms have also been recovered from horses and often identified as haemolytic variants of A. equuli, some taxonomic clarification for haemolytic organisms from pigs and horses is needed.

It has been the purpose of the present work firstly to examine the morphological, cultural, biochemical and fermentative characters of a number of strains of A. equuli recovered from pathological materials of equine and porcine origin and from samples taken from normal horses; secondly, to determine the antigenic characteristics of A. equuli and, possibly, to relate them to other properties of the organism; and lastly to compare the haemolytic strains from horses and pigs in the work with the haemolytic porcine strains identified as Actinobacillus suis.

GENERAL INTRODUCTION

The organism now known as Actinobacillus equuli was first described by Meyer (1910) who isolated a highly pleomorphic Gram-negative bacillus in a case of purulent nephritis in a horse in South Africa. In a report covering the years 1908-1909, he named the organism isolated from the abscesses in the kidneys Bacillus nephritidis equi while admitting the same organism was observed as early as 1902 by Theiler who conducted some experiments on the study of purulent nephritis in horses in the Transvaal, but failed to demonstrate the further proof of its importance as a specific cause of nephritis in horses.

M'Fadyean and Edwards (1919), without making any claim to priority, mentioned that in March 1901 the same organism described by Meyer had been isolated by M'Fadyean from the pus in a case of pyaemic nephritis secondary to a punctured foot in a horse in London. They also stated that the most striking cultural character of the bacillus was the viscosity of its broth cultures and that the cultures died out before the organism could be further studied.

Magnusson (1917), finding the organism in foals in Sweden and not recognizing it as the same as that described by Meyer, named it Bacterium viscosum equi.

Many names have been encountered for this species of micro-organism and indeed few other species of

bacteria have undergone such kaleidoscopic changes in nomenclature. The variety of synonyms which have been given to Actinobacillus equuli include Bacillus nephritidis equi (Meyer, 1910), Bacterium viscosum equi (Magnusson, 1917), Bacillus equuli (van Straaten, 1918), Bacillus pyosepticus equi (de Blieck and van Heelsbergen, 1919), Bacterium pyosepticum viscosum (Miessner, 1921), Bacterium pyosepticum viscosum equi (Lütje, 1921), Bacterium pyosepticum (Miessner and Berge, 1922), Bacterium equi (Weldin and Levine, 1923), Bacillus pyosepticus (Clarenberg, 1925), Eberthella viscosa (Snyder, 1925), Shigella equi (Weldin, 1927), Shigella viscosa Snyder (Bergey, Harrison, Breed, Hammer and Huntoon, 1930), Shigella equirulis de Blieck and van Heelsbergen (Edwards, 1931), Shigella equuli van Straaten (Jansen, 1941; Dimock, Edwards and Bruner, 1947a) and Shigella viscosum equi (Belonje, 1952). Sakazaki and Watanabe in 1956 proposed that "Bacterium viscosum equi (Shigella equirulis) should be transferred from the family Enterobacteriaceae to Parvobacteriaceae, and classified, perhaps, as belonging to the Pasteurella D group according to Ochi (1934) because of its similarity of morphological, cultural and biochemical behaviour and the sensitivity to penicillin to those of Pasteurella multocida". More recently it was included in the genus Achromobacter as Achromobacter equuli (Cowan and Steel, 1961). Breed, Murray and

Smith (1957) in the 7th edition of Bergey's Manual of Determinative Bacteriology considered it Actinobacillus equuli which had been previously proposed by Haupt (1934).

Generally, the previous workers who gave special species epithets to this microorganism were impressed by the viscosity of its culture (viscosa, viscosum), by its host character (equuli, equi) and by the nature of the disease developed in the host (nephritidis, pyosepticus, pyosepticum), thus species names appearing in the literature are either simple expressions of one of its characters (e.g. Bacillus equuli, Bacterium pyosepticum, Shigella viscosa etc.) or combinations of those features (e.g. Bacterium nephritidis equi, Bacterium viscosum equi, Bacterium pyosepticum viscosum equi etc.).

A. equuli has been identified as an important cause of neonatal death in foals in most countries of the world and may be responsible for as high as 25 per cent. of all infections in newborn foals (Seddon and Albiston, 1965; Blood and Henderson, 1974). Newborn foals are quite vulnerable to bacterial infections. Three microorganisms, A. equuli, haemolytic streptococci and Escherichia coli, are responsible for the majority of infections and deaths of young foals (Magnusson, 1919; M'Fadyean and Edwards, 1919; Lütje, 1922b; Dimock, Edwards and Bruner, 1947a,b; Miller, 1950; Platt,

1973).

Magnusson (1919) recorded an interesting account of investigations on 314 carcasses of foals, 236 of which died of joint-ill. He noted that the three predominant causes of infection were streptococcus, A. equuli and E. coli, which accounted for 31.8 per cent., 30.9 per cent. and 27.1 per cent. respectively. No less than 56 out of 73 cases of joint-ill ascribed to A.equuli became ill on the first day of life and more than half of the foals died within 2 days showing symptoms of acute septicaemia.

According to data compiled by Dimock et al. (1947a), A. equuli infection caused the deaths of 31.5 per cent. of 810 foals and streptococcal infections were responsible for death of 25.7 per cent. of the same group. During the first week after birth, fatal cases of A. equuli infection exceeded those due to streptococci by a ratio of 2.6 to 1. For foals dying at ages of 8 to 30 days, the cause of mortality was reversed with deaths from streptococci exceeding those from A. equuli by a ratio of 3.6 to 1. Uncomplicated septicaemia due to A. equuli was characteristically a disease of the very young foal.

Miller (1950) reported on the basis of two years survey that A. equuli infection accounted for the death of 28.6 per cent. of 70 foals and streptococcal infections caused 17.1 per cent. of these deaths.

More recently Platt (1973) described an account of his findings in 61 foals with generalised infections subjected to post mortem examination at the Equine Research Station (Newmarket, England) during 1967-1972. E. coli infection accounted for 42.6 per cent. of these deaths and A. equuli infection for 29.7 per cent. Salmonella typhimurium infection caused the death of 13.3 per cent. of the same group while haemolytic streptococcal infection accounted for only 9.8 per cent.

A. equuli infection in foals most often takes the form known as "sleeper" or "sleepy foal", an acute septicaemia of newborn foals which is one of the most common diseases to which foals are subjected at, or shortly after birth (Udall, Fincher and Gibbons, 1929; Dimock et al., 1947b; Farrelly and Cronin, 1949; Report, 1949; Littlejohn, 1959; Seddon and Albiston, 1965; Blood and Henderson, 1974). The term "sleepy" has been applied to affected foals on account of the extreme prostration, listlessness, lethargy and stupor which are produced by the condition (Farrelly and Cronin, 1949; Cottew and Ryley, 1952; Leader, 1952).

The disease has also been called joint-ill (M'Fadyean and Edwards, 1919; Magnusson, 1919; Adsersen, 1920b; Dimock, Edwards and Bullard, 1928; Miessner and Köfer, 1935; Hirato, 1939; Lesbouryies, 1945; Gunning, 1947; Miller, 1950; Maguire, 1958; Hutchinson, 1956), viscosum infection (Magnusson,

1931), shigellosis (Brudnjak and Zelenka, 1961; Jubb and Kennedy, 1970; Blood and Henderson, 1974), pyosepticaemia of the new-born (Adsersen, 1920a; Miessner and Berge, 1922; Farrelly, 1951; Cottew and Ryley, 1952), actinobacillosis (Doll, 1963; Knight, 1972) and omphalophlebitis, dummy foal and wanderers (Doll, 1949 and 1963; Knight, 1972). More recently Bruner and Gillespie (1973) proposed that "the infection be named equulosis, a designation based on the specific part of the binomial".

The disease in foals is characterised by sudden onset, extreme prostration, short duration and death (Knight, 1972; Blood and Henderson, 1974). In 69 per cent. of A. equuli infections reported by Dimock et al. (1947a) the foals died within the first four days of life. In new-born foals the usual symptoms are dullness, weakness and inappetence. Lameness may be present but often the foal is too weak to stand. Some foals show symptoms of nervous disorder, continually walking round the stall and refusing to suckle. Occasional foals show severe abdominal pain in the early stages of the disease. Most foals pass a small amount of urine at frequent intervals, accompanied in many cases by a certain amount of irritation (Littlejohn, 1959). Foals that survive the acute, febrile phase develop arthritis with swollen joints and lameness within

1 or 2 days (Blood and Henderson, 1974; Jubb and Kennedy, 1970). Death usually occurs in these more protracted cases during the period between the second and seventh days (Magnusson, 1919; Dimock et al., 1947a,b; Miller, 1950; Blood and Henderson, 1974).

In older foals the disease is characterised by sudden prostration, fever and increased pulse rate, death usually occurring in a few hours (Dimock and Edwards, 1932).

Aborted foals and those dying acutely of septicaemia usually do not have distinctive lesions. Foals that die within 24 hours of the onset of illness show septicaemia and severe enteritis but there is no involvement of the kidney (Dimock et al., 1947a,b; Littlejohn, 1959; Jubb and Kennedy, 1970). The adrenals are enlarged in the majority of cases and dark red in colour (Magnusson, 1919; du Plessis, 1963). Occasionally reddish-grey areas of grey hepatisation in the lungs are observed in these cases (du Plessis, 1963).

In foals which died at 2 to 3 days of age the lesions are more marked. The macroscopic picture of these cases presents, in addition to septicaemic changes, typical pin-head to pea-sized abscesses scattered throughout the cortex of the kidney (Magnusson, 1919; Dimock and Edwards, 1932; Littlejohn, 1959; Bruner and Gillespie, 1973). In the majority of

cases the joints of the legs are affected (Magnusson, 1919; Dimock et al., 1947a,b; Belonje, 1952). The joint lesions range from a slight increase in synovial fluid and congestion of the joint capsule to a purulent arthritis involving the joint cavity and tendon sheaths, with a great accumulation of fluid and extreme swelling (M'Fadyean and Edwards, 1919; Magnusson, 1919; Dimock et al., 1947a,b; Blood and Henderson, 1974). In practically all cases, save the most acute ones, the principal changes are found in the kidneys. The cortical abscesses, which are readily visible to the naked eye when the capsule of the kidney is stripped, are the principal changes found in these organs. They vary from a pin point to a pin-head in size. The condition is primarily a glomerulonephritis followed by suppuration and necrosis (Belonje, 1952; Jubb and Kennedy, 1970). Similar microabscesses may be found in many organs especially in the lungs. Some foals show purulent pneumonia (Dimock et al., 1947a,b; Carter, Marshall and Jolly, 1971).

The fact that this infection was found in aborted foetuses (Dimock et al., 1947a,b; Monteverde, 1949) and lesions observed in the lungs and adrenals of the foals which died within 24 hours of birth (du Plessis, 1963) definitely indicates a prenatal origin, but much of the evidence obtained from post mortem

studies of foals points to a postnatal origin of the infection (Dimock et al., 1947a,b). Attempts to reproduce the infection as a prenatal disease in foals by introducing organisms into the uteri of three mares proved unsuccessful (Dimock et al., 1947a,b). It is thought that prenatal infection, although uncommon, is possible by means of migrating Strongylus vulgaris larvae carrying the organisms from the dam's intestine to her blood stream and thence across the placenta to the foetus in the womb (Basset and Moulin, 1933; Dimock et al., 1947a,b; Davies, 1960; Merchant and Packer, 1967). Maguire (1958) was of the opinion that the acute infection in new-born foals is undoubtedly a prenatal infection and that 90 per cent. of foals born to carrier mares are diseased at birth and will reveal typical signs of infection at a very early age.

Many adult horses including brood mares are carriers of A. equuli. It is frequently found in the tonsillar region and in the intestines but rarely in the genital tract. A. equuli has been found in verminous aneurysms in horses that showed no evidence of infection elsewhere (Dimock et al., 1947a,b). It was theorized that the organism may be carried to the site by Strongylus vulgaris larvae. Noting that positive precipitin reactions were often observed in normal horses, Meyer as early as 1910 stated that A. equuli might be a saprophyte which under certain conditions becomes

pathogenic. Indeed many workers proved the commensal nature of A. equuli in the digestive tract of horses. In 1923 Laudien isolated A. equuli from the intestinal contents of 13 horses. Jarmai (1929) noted that septicaemia caused by A. equuli in older foals and adult horses usually was preceded by a respiratory disorder, and concluded that A. equuli was a secondary invader which attacked animals only when their resistance was lowered by some other agents. Assuming that A. equuli probably was distributed widely and was commonly present in normal horses, he took cultures from the tonsillar region of 67 normal horses and isolated A. equuli from 49 of them. Dimock and Edwards (1932) isolated the organism from the tonsillar region in 10 of 12 horses which had been destroyed or which had died from infections other than A. equuli. In 1954 Cottew and Francis recovered 30 strains of A. equuli from mouth and cervical swabs from 59 horses varying in age from one week to 25 years. Considering the almost constant presence of the bacterium in the mouth, in the tonsillar area and in the intestinal tract and faeces, any place where horses are kept would be contaminated with the organism.

Postnatal infection is believed to be the most common method of infection, the organism entering via the mouth, respiratory tract or umbilicus. Probably oral infection is the most common and infection via

the umbilicus least common so that local lesions are rarely found at that point. The organisms recovered from the dead tissues of foetus or foal are exactly the same as those which are frequently recovered from the so-called "dirty mare", which has had a uterine discharge before or during the covering season (Miller, 1950). Thus, infection in the newly born foal may occur from the dam during suckling or licking.

A. equuli is considered to be an opportunist in young foals, and is particularly likely to develop in those which are congenitally defective in some respect. This infection is more common in weak, debilitated, or incompletely developed foals with defensive mechanisms unprepared for the immediate environment (Doll, 1963). Such foals are predisposed to infection with many opportunistic pathogens. Belonje (1952) was of the opinion that nutritional factors may be concerned in the development of the disease and a high proportion of the foals which developed the disease were small and undersized. Platt (1973) reported his findings in 61 cases of acute generalized infection in the foal with particular reference to the types of organism concerned and the role of predisposing factors. Factors which reduced colostral intake including deliberate deprivation to prevent haemolytic disease, death of dam, delayed and feeble suckling in weak or premature foals, deformities of the limbs or mouth, and the

development of the convulsive syndrome or other behavioural abnormality soon after birth, played crucial roles in the pathogenesis of infections during the first week of life and 37 out of 44 infected foals up to one month old were abnormal in some respect. Any factor which interferes with prompt and vigorous suckling is likely to reduce the amount of γ-globulin intake since the transfer of maternal antibody is entirely dependent upon the colostrum intake (Jeffcott, 1971).

Natural cases of infection in adult horses caused by A. equuli have been observed from time to time. Although the organism has been recognized as the most common cause of embolic suppurative nephritis in horses (Hutyra, Marek and Manninger, 1938; Jubb and Kennedy, 1970), it has seldom been of pathological significance in adult horses.

Stricker and Grunert (1956) described an acute septicaemic disease of high mortality which had occurred in adult horses in several districts of Slovakia. A. equuli was isolated from 40 per cent. of samples of liver, lung and spleen material from 30 horses which had died of septicaemia, characterized clinically by high fever, diarrhoea, staggering gait and hyperaemia of the mucosae.

Áldásy and Süveges (1964) reported their findings on 107 horse carcasses of which 35 were confirmed

bacteriologically as cases of A. equuli septicaemia. Their observations suggest that it is brought about by devitalizing factors viz. heavy work introduced suddenly after a long rest, lack of exercise, poor nutrition and viral infection etc.

Magnusson (1919) reported a case of septicaemia ascribed to A. equuli in a 6-year-old mare which had been injured by the stallion in the act of covering, and subsequently died of a fatal septicaemic infection characterized by necrotic enteritis, pulmonary abscesses and erosions in the lips of the vulva and perineum.

Sugano, Kobayashi and Yamagiwa (1949), on the basis of pathological studies on the bacterial infections associated with infectious anaemia, emphasised the significance of the mixed infection with A. equuli in the acute form of infectious anaemia.

Tiedge (1936) claimed that the malignant course which strangles often runs is due chiefly to the superimposition of an infection with A. equuli or Salmonella enteritidis.

A. equuli has been identified as the cause of abortion in mares (Lütje, 1922b; Langhoff, 1923; Dimock and Edwards, 1932; Britton, 1945; Cordier, Rigaud and Ounais, 1954; Siegmund, 1973) although the frequency is very low. Mares which are caused to abort by this infection are not ill, and the organism does not persist for long in the uterus after abortion.

A mare rarely has two foals that die of this infection and after giving birth to a foal infected with A. equuli she recovers as rapidly as a mare that has given birth to a normal foal (Dimock and Edwards, 1932). However, the same mare may abort in successive pregnancies, a fact which suggests that the uterus can be re-infected from some endogenous asymptomatic focus. The organism has been isolated from the bone marrow of an aborted equine foetus (Monteverde, 1949).

On the other hand Zakopal and Nesvadba (1968) claimed that in an outbreak of A. equuli septicaemia among the mares of a breeding stud, no abortions occurred nor did the foals that nursed their dams develop the disease.

A. equuli has also been identified as the cause of endocarditis in horses (Svenkerud and Iverson, 1949; Innes, Berger and Francis, 1950; Vallée, Tinelli, Guillon, Le Priol and Tran Cuong, 1974). Innes et al. (1950) described a case in a horse of subacute bacterial endocarditis associated with A. equuli, and characterized pathologically by exuberant, friable vegetations involving the semilunar valves and by a sequel of pulmonary embolism, while Larsen (1974) recorded the occurrence of this organism in chronic alveolar emphysema in horses in Denmark.

A. equuli was isolated from pus from a nine-year-old horse with chronic funiculitis (Jansen, 1941; Hartog,

1941), and post-partum mastitis ascribed to A. equuli has been recorded (Report, 1971).

Weidlich (1955) recorded a case of rhinitis and meningitis in a 3-year-old stallion. The disease was characterized pathologically by inflammation and ulceration of the serosa of the nose, an abscess of the dura mater and an inflammation of the adjacent pia mater.

With the discovery of A. equuli infection in horses in South Africa, the disease due to this organism has been reported as an important cause of neonatal death and infecting older horses in many other parts of the world including Argentina (EcKell, Crliacz and Garagarz, 1949; Monteverde, 1949), Australia (Cottew and Ryley, 1952; Bain, 1954; Seddon and Albiston, 1965), Brazil (de Fretas and Salles Gomes, 1959), Czechoslovakia (Stricker and Grunert, 1956; Mráz, Zakopal and Matousek, 1968; Zakopal and Nesvadba 1968), Denmark (Adsersen, 1916, 1919, 1920a; Larsen, 1974), France (Gourvitch, 1931; Lesbouyries, 1945; Pigoury, Benazet, Courtin, Charny, Michel and Chabassol, 1959; Vallée et al., 1974), Germany (Miessner, 1921; Miessner and Berge, 1922; Reinhardt, 1922; Otto, 1922; Lütje, 1922a,b; Langhoff, 1923; Jarmai, 1929; Miessner and Köfer, 1935; Weidlich, 1955), Great Britain (M'Fadyean and Edwards, 1919; Gunning, 1947; Report, 1949; Innes et al., 1950;

Miller, 1950; Farrelly, 1951; Baker, 1972; Platt, 1973), Holland (van Straaten, 1918; de Blieck and van Heelsbergen, 1919; Jansen, 1941), Hungary (Gunther, 1956; Héjj, 1956; Széky, 1960; Áldásy and Süveges, 1964), Italy (Ubertini, 1933), Ireland (Maguire, 1958), Japan (Hirato, 1939; Sugano et al., 1949; Sakazaki and Watanabe, 1956), New Zealand (Hutchinson, 1956; Bloomberg, 1963), Norway (Svenkerud and Iverson, 1949), Poland (Dabrowski and Lorkiewicz, 1949; Parnas and Lorkiewicz, 1950; Dabrowski and Woloszyn, 1954), South Africa (Meyer, 1910; Belonje, 1952; Littlejohn, 1959; du Plessis, 1963), Sweden (Magnusson, 1917, 1919, 1920; Rubarth and Henricsen, 1941), Tunisia (Cordier, Rigaud and Ounais, 1952, 1954), the U.S.A. (Snyder, 1925; Dimock et al., 1928; Udall, Fincher and Gibbons, 1929; Bullard, 1930; Dimock and Edwards, 1932, 1933; Dimock, 1935; Hafmann, 1940; Britton, 1945; Dimock et al., 1947a,b; Doll, 1949; Doll and McGee, 1951), the U.S.S.R. (Gourvitch, 1931) and Yugoslavia (Brudnjak and Zelenka, 1961).

A. equuli infection has also been reported on several occasions from pigs with acute generalised infection, but it was not until nearly 15 years after the original description of A. equuli by Meyer (1910) that a case of septicaemia caused by this organism in a 16-day-old piglet was recorded by Clarenburg (1925) in Germany. In 1923 he isolated the microorganism

resembling the bacillus of joint-ill in foals from the spleen, liver and kidneys of the piglet.

Lütje (1924) reported a single case of a newly-purchased pig dying after only a few hours' illness and later 4 cases in piglets which died suddenly of a septicaemia due to A. equuli infection (Lütje, 1925).

Magnusson (1931) described no less than five cases of a septicaemia due to Actinobacillus infection in suckling pigs in Sweden. He examined five cases in the same litter, in which the most characteristic changes proved to be purulent polyarthritis and miliary abscesses with haemorrhages in the kidney and stated that the organisms which he isolated were culturally almost identical with A. equuli of the foal. He also emphasized the fact that the isolated organisms were pathogenic for young pigs when injected intravenously and intraperitoneally, while foal isolates were not pathogenic for pigs. From four cases of focal nephritis he also recovered an haemolytic actinobacillus which agreed in most characters examined with Bacillus polymorphus suis originally described by Degen (1907). He stated that this organism differed from A. equuli in its haemolytic and serological properties, in its failure to form sticky colonies and in its virulence for experimental animals.

Since then haemolytic and non-haemolytic actinobacilli have been reported as a cause of disease in the pig. The non-haemolytic actinobacilli from pigs have been identified as A. equuli (Magnusson, 1931; Edwards and Taylor, 1941; Jansen, 1941; Ashford and Shirlaw, 1962; Zimmermann, 1964, 1965; Jones and Simmons, 1971; Windsor, 1973) but the toxonomic status of haemolytic actinobacilli is still uncertain (Wetmore, Thiel, Herman and Harr, 1963; Bouley, 1966; Mráz, 1968; Cutlip, Antower and Zinober, 1972; Ross, Hall, Orning and Dale, 1972; Windsor, 1973; Bell, 1973). van Dorssen and Jaartsveld (1962) and Zimmermann (1964) claimed that the characters of the haemolytic actinobacilli from pigs were quite distinct from those of A. lignieresi and A. equuli and named them A. suis. On the other hand, Mráz (1968) took the view that the strains of van Dorssen and Jaartsveld (1962) and those of Wetmore et al. (1963) represented haemolytic variants of A. equuli.

Diseases in pigs from which haemolytic and non-haemolytic actinobacilli have been isolated include arthritis (Magnusson, 1931; van Dorssen and Jaartsveld, 1962; Wetmore et al., 1963), endocarditis (Terpstra and Akkermans, 1955; Ashford and Shirlaw, 1962; Jones and Simmons, 1971), enteritis (Zimmermann, 1964), hepatitis (van Dorssen and Jaartsveld, 1962),

meningoencephalitis (Terpstra and Akkermans, 1955), metritis (Edwards and Taylor, 1941), nephritis (Degen, 1907; Magnusson, 1931; Hutyra et al., 1938), pericarditis (van Dorssen and Jaartsveld, 1962), pneumonia (Terpstra and Akkermans, 1955; van Dorssen and Jaartsveld, 1962) and septicaemia (Clarenburg, 1925; Lütje, 1924, 1925; Magnusson, 1931; Ubertini, 1933; Jansen, 1936, 1941; Zimmermann, 1964; Bouley, 1966; Cutlip et al., 1972; Windsor, 1973; Mair, Randall, Thomas, Harbourne, McCrea and Cowl, 1974).

Cutlip et al. (1972) reported the isolation of a haemolytic actinobacillus from one of 3 pigs which died of an acute septicaemic disease. At the time of death each pig had large irregular erythematous areas in its skin that might have been confused with lesions of swine erysipelas. More recently haemolytic actinobacilli have been recovered from piglets with a purpura-like condition showing dark greasy scabs on the upper lips and purple oedematous swellings of the forelegs (Bell, 1973; Windsor, 1973).

Generally, swine actinobacillosis is characterised as an acute septicaemia of young pigs and less frequently of pigs over 3 months of age (Zimmermann, 1965; Cutlip et al., 1972; Windsor, 1973; Mair et al. 1974). After investigating actinobacillosis in a litter of pigs and reviewing reports on similar infections,

Windsor (1973) expressed his opinion that, "the younger the animal, the more rapidly death ensues. With increasing age animals can more easily overcome the septicaemia although some usually remain unthrifty".

Septicaemia of older pigs was often associated with other general infections such as swine fever, enzootic pneumonia and swine plague (Ubertini, 1933; Terpstra and Akkermans, 1955; Sato and Sugimura, 1955; Hézz and Szabo, 1957; Zimmermann, 1965).

Sato and Sugimura (1955) described their investigations in mixed bacterial infections in swine fever in Japan. Haemolytic actinobacilli were isolated from 68 of 560 pigs which had been submitted to the potency test for swine fever vaccine. They suggested that these organisms might be of some importance in association with swine fever. Later Hézz and Szabo (1957), on the basis of their findings on swine fever infection in Hungary suggested that if A. equuli septicaemia is seen then swine fever should be suspected.

Wetmore et al. (1963) reported an interesting account of their findings during the course of studies on the effects of irradiation in swine. They isolated haemolytic actinobacilli from the blood and/or synovial fluids of the inflamed hocks of 4 adult pigs and 1 piglet, and claimed that the circumstances

which produced the disease provides ancillary evidence of the occurrence of actinobacilli as a commensal parasite of normal swine.

Actinobacillus infection complicated with actinomycosis in pigs has been reported recently in the U.S.S.R. by Nelyubin (1967) who identified the organism isolated as A. lignieresi.

Ross et al. (1972) described four strains of an haemolytic, urease positive, Gram-negative microorganism isolated from vaginal exudates of postparturient sows in the U.S.A. They stated that these swine isolates should be classified in the genus Actinobacillus on the grounds of their growth requirements and morphological, biochemical and antigenic characteristics although they distinguished them from the established species of the genus. They regarded these swine actinobacilli as a new species but they did not propose a species name because of the current lack of clear guidelines for speciation within the genus Actinobacillus.

The geographical distribution of swine actinobacillosis is extensive with workers having reported its occurrence in Czechoslovakia (Mráz, 1968), France (Bouley, 1966), Germany (Clarenburg, 1925; Lütje, 1924, 1925; Zimmermann, 1964, 1965), Great Britain (Jones and Simmons, 1971; Bell, 1973; Windsor, 1973; Mair et al., 1974; Report, 1975), Holland (Jansen, 1941; Terpstra and Akkermans, 1955; van Dorssen and

Jaartsveld, 1962), Hungary (Hézz and Szabo, 1957), Italy (Ubertini, 1933), Kenya (Ashford and Shirlaw, 1962), Sweden (Magnusson, 1931), the U.S.A. (Edwards and Taylor, 1941; Wetmore et al., 1963; Cutlip et al., 1972) and the U.S.S.R. (Nelyubin, 1967).

The occurrence of A. equuli infection in animals other than horses and swine has also been reported from time to time. du Plessis, Cameron and Langen (1967) described cases of focal necrotic pneumonia and rumeno-enteritis caused by A. equuli infection in 5 Afrikaner calves. Infection by this organism was reproduced experimentally in a calf which developed diarrhoea and showed some lesions resembling those in natural cases.

Osbaldiston and Walker (1971) supported the evidence of du Plessis et al. (1967) that A. equuli could be an enteric pathogen of calves. They described three separate cases of calf scours characterized by necrotic ruminitis, inflammation of the intestine and pleuro-pneumonia, which they had diagnosed as actinobacillary enteritis caused by A. equuli.

Actinobacillus spp. have not been reported in studies of the normal flora of the bovine intestine (Smith and Orcutt, 1925; Smith, 1960; Smith, 1965). Phillips (1960) isolated actinobacilli from the ruminal contents of 31 of 306 healthy adult cattle. He suggested that the commensal nature of Actinobacillus

in the rumen is consistent with the generally held view that the organism enters the tissue through wounds of the skin and of the oral and ruminal mucous membrane.

Mohanty and Singh (1970) described a case of actinobacillary nephritis in a male buffalo in India. No bacteriological examination of material taken from the case was made and whether the diagnosis was correct or not remains uncertain.

Vallée, Durieux, Durieux and Virat (1960) described an outbreak of persistent suppurative dermatitis in lesions of the interdigital spaces and lower lip associated with A. equuli and staphylococcus infection in a dog.

Infection of primates with A. equuli has been reported. Moon, Barnes and Higbee (1969) described 2 cases of septic embolic actinobacillosis ascribed to A. equuli in a squirrel monkey (Saimiri sciureus) and a spider monkey (Ateles panuscus). The disease in monkeys, characterized by widespread bacterial embolism and embolic suppurative nephritis, was similar to the disease caused by A. equuli in foals. They considered that the "stress" resulting from shipping predisposed these monkeys to the disease.

A. equuli has been considered to be pathogenic for the rabbit and hare (Ubertini, 1933; Brudnjak, 1948; Vallée, 1959; Biancardi, 1960). Ubertini (1933)

presented the first record of the isolation of the organism from pus of a wild rabbit with a subcutaneous abscess in the leg and an embolic pulmonary abscess. An abscess was reproduced in a rabbit by subcutaneous inoculation of the organism. A. equuli has been isolated from a submaxillary abscess (Brudnjak, 1948), a case of coccidial hepatitis and a pneumonic lung lesion in a rabbit (Vallée, 1959). Biancardi (1960) recorded a case of actinobacillary septicaemia in a rabbit which revealed punctiform haemorrhages in the peritoneum, haemorrhagic infarcts in the lung, enlarged spleen and subserous haemorrhages in the intestine.

Arseculeratne (1961) reported the isolation of an organism, which appears to belong to the genus Actinobacillus, from a naturally occurring infection in 3 laboratory rabbits. A large multilocular non-calcified abscess exuding thick yellowish pus containing no granules was found in lesions of tarsal joints and a Gram-negative, non-motile bacillus, which was regarded as distinct from A. lignieresi, was isolated from one of them. The following year, the name Actinobacillus capsulatus was proposed for this isolate because its properties, including pathogenicity, are sufficiently distinctive to regard it as a hitherto undescribed species of Actinobacillus (Arseculeratne, 1962).

The organism isolated from natural cases of ovine

epididymitis in Queensland, Australia was considered to be most closely related to the genus Actinobacillus (Baynes and Simmons, 1960). The new species name Actinobacillus seminis was given by Baynes and Simmons (1960) for this biochemically inert Gram-negative, non-motile bacterium which for maximal growth required lowered oxygen tension and serum enrichment. Since then it has been reported with increasing frequency in Australia (Galloway, 1966; Simmons, Baynes and Ludford, 1966; Baynes and Simmons, 1968), South Africa (Worthington and Bosman, 1968; van Tonder and Bolton, 1968) and the U.S.A. (Livingston and Hardy, 1964). It has also been isolated from cases of polyarthritis and posthitis in sheep in Western Australia (Watt, Bamford and Nairn, 1970) and from a case of epididymitis in a young Friesland bull in South Africa (van Tonder and Bolton, 1970). In New Zealand, however, Ekdahl, Money and Martin (1968) isolated from cases of epididymitis in rams an Actinobacillus-like organism, the character of which was not identical with those of the Australian strains.

The organism referred to as Actinobacillus seminis was perhaps wrongly considered to belong to the genus Actinobacillus (Gumbrell and Smith, 1974). Phillips (1974) commented in the 8th edition of Bergey's Manual of Determinative Bacteriology that Actinobacillus seminis (Baynes and Simmons, 1960) isolated from cases

of ovine epididymitis should be excluded from the genus Actinobacillus because of their failure to ferment any of a wide range of carbohydrates. Gumbrell and Smith (1974) compared the guanine-cytosine base ratio of a group of Actinobacillus-like organisms isolated from ovine genital lesions with that of known type strains of A. lignieresi and A. equuli in order to demonstrate some sort of relationship between them. They concluded that the ovine isolates considered as probable or confirmed "A. seminis" are not of one species, and possibly not of one genus as difference of % GC of 5 usually implies at least a species difference (Jones and Sneath, 1970).

Part I: The biochemical and cultural characters of *Actinobacillus equuli* from various sources

1. INTRODUCTION

Finding the organism now known as Actinobacillus equuli from the viscid pus of kidney abscesses and lung nodules in a horse, Meyer in 1910 described the characters of the bacillus which caused specific purulent nephritis. He defined the organism as a Gram-negative, non-motile, non-sporulating markedly pleomorphic bacillus which is indole negative, does not produce hydrogen sulphide, liquefies gelatin and ferments glucose, mannose, galactose, fructose, maltose, dulcitol and mannitol without gas production. Although the limited number of biochemical and cultural characters of the organism were defined, some prominent properties of the bacillus such as the pleomorphic nature of the organism, its low viability and the stickiness of its colonies in culture media, were well pointed out from the beginning.

In the course of time the characters of the organism have been more widely defined by subsequent workers (Magnusson, 1919 & 1931; Snyder, 1925; Dimock, Edwards and Bullard, 1928; Edwards, 1931; Ubertini, 1933; Haupt, 1934; Hirato, 1939; Cottew and Francis, 1954; Sakazaki and Watanabe, 1956; Vallée et al., 1963; Phillips, 1966; Windsor, 1973). However, it became more apparent that considerable variation existed in the properties of the organism. The more investigators worked with the organism, the more variations in

characters of the bacillus, particularly in fermentative reactions, were noted.

Early fermentative and biochemical studies on the organism were often conducted on a small number of strains (Ashford and Shirlaw, 1962; Baker, 1972; Carter, Marshall and Jolly, 1971; Haupt, 1934; Magnusson, 1931; Osbaldiston and Walker, 1972; Wetmore et al., 1963; Zimmermann, 1964), tested in a very limited range of carbohydrates (Baker, 1972; Cowan and Steel, 1970; Meixner, 1931; Moon, Barnes and Higbee, 1969; Osbaldiston and Walker, 1972; Windsor, 1973; Zimmermann, 1964) and in a limited number of biochemical activities (Cottew and Francis, 1954; Dimock, Edwards and Bullard, 1928; Jones and Simmons, 1971; Neter, 1942; Snyder, 1925; Ubertini, 1933; Zimmermann, 1964). Thus, satisfactory comparisons between the results of previous workers are not always possible.

While defining the genus Actinobacillus Brumpt as "Gram-negative, non-motile, non-acid-fast rods, sometimes occurring in long chains or in unjointed filaments" and "in lesions in the animal body no mycelium is formed, but at the periphery finger shaped cells or clubs may be visible," Wilson and Miles (1964, p.514; 1975, p.544) also expressed the view that the systematic position of this group is doubtful. Cowan (1974, p.95) described the uncertain taxonomic position of this group of

bacteria as "Actinobacillus is made up of species for which no obvious home can be found".

Six members of the genus Actinobacillus were described in the 6th edition of Topley and Wilson's Principle of Bacteriology, Virology and Immunity. The names of the organisms are: A. lignieresi, A. actinomycetemcomitans, A. actinoides, A. moniliformis, A. piliformis and A. equuli. The resemblance between A. actinoides and the organism commonly known as Streptobacillus moniliformis (syn., Streptothrix muris ratti; Actinomyces muris; Actinobacillus muris) has been noted (Dienes and Edsall, 1937) but more information is needed before judgement can be made. The name Actinobacillus piliformis was tentatively given by them for the organism, Bacillus piliformis, that was described by Tyzzer (1917) as responsible for a disease of Japanese waltzing mice, by Gard (1944) for summer diarrhoea in albino mice, and Allen, Ganaway, Moore and Kinard (1965) for enteritis in rabbits characterized by fulminating watery diarrhoea believed to be indistinguishable from that of Tyzzer's disease in mice. The biology and natural history of B. piliformis remains virtually unknown. Hence its taxonomic position cannot be determined unless more information is provided.

Wetmore et al. (1963) compared haemolytic isolates recovered from swine and foal with known strains of A. lignieresi and A. equuli. Finding no remarkable

differences among swine and foal isolates and established strains of A. lignieresi and A. equuli, except for minor and variable differences in fermentation of melibiose, mannitol and salicin and in the production of haemolysis, they proposed that the specific epithet equuli be dropped and that strains heretofore identified as A. equuli be reclassified in the species A. lignieresi. Furthermore, Wilson and Miles (1964, 1975) are of the opinion that the taxonomic position of A. equuli is uncertain. On the other hand, Vallée, Thibault and Second (1963) regarded A. lignieresi as a different species from A. equuli on the basis of the ability of the latter to ferment lactose and to produce hydrogen sulphide. Bohácek and Mráz (1967) differentiated two species on the different GC content of their DNA. In the 8th edition of Bergey's Manual of Determinative Bacteriology, Phillips (1974) tabulated the differential characters of A. lignieresi and A. equuli. The two species would be separated on the failure of A. lignieresi to ferment raffinose and trehalose, to liquefy gelatin, to hydrolyse sodium hippurate and to form a broth deposit and of A. equuli to reduce methylene blue.

However, evidence has been accumulating that A. equuli and other closely related, but not identical, organisms which are haemolytic on blood agar and mostly fail to ferment mannitol, produce a variety of syndromes in horses (Ubertini, 1933; Svenkerud and Iverson, 1949;

Mráz, Zakopal and Matousek, 1968; Carter, Marshall and Jolly, 1971; Hughes and Murphy, 1972; Larsen, 1974) and pigs (Wetmore et al., 1963; Mráz, 1968; Cutlip et al., 1972; Rose et al., 1972; Bell, 1973) and are not uncommonly isolated following the culture of swabs obtained from trachea, pharynx, mouth, nose and faeces of horses (Cottew and Francis, 1954; Hughes and Murphy, 1972). So some taxonomic clarification for these groups of organisms is needed. It is the purpose of this part of the work to examine the morphological, cultural, biochemical and fermentative characters of 132 strains of A. equuli recovered from pathological material of equine and porcine origin and from material taken from normal horses, and of 6 strains of A. equuli obtained from the National Collection of Type Cultures.

2. MATERIALS AND METHODS

(a) Media and reagents

Except where otherwise indicated the media and reagents used for the characterisation of A. equuli were prepared as described by Cowan and Steel (1965).

(b) Sources of strains of A. equuli

One hundred and thirty-eight strains of A. equuli were examined in this section of the work of which six were obtained from the National Collection of Type Cultures (no. 3365, 8529, 8644, 8794, 8987 and 9435). All strains studied in this investigation were available in the Culture Collection of the Royal (Dick) School of Veterinary Studies, Edinburgh. The information regarding the strains used in this study is summarised in Table 1 and individually in Appendix A.

Table 1

Sources of strains used in the present study

	Pathological material	Normal animal	Unknown origin	NCTC strains	Total
Horse	16	101	5	6	128
Pig	9	0	1	0	10
Total	25	101	6	6	138

(c) Preservation of strains

All strains were received in lyophilised form or had been stored in frozen form at -50°C. Immediately after receiving the organism it was subcultured on blood agar and in glucose broth. Representative colonies were transferred into cooked meat medium (Oxoid) and incubated for 24 hours. The organisms maintained in this medium were stored in the dark at room temperature and used for day-to-day stock culture. The stock cultures were subcultured at two- to three-week intervals. A careful watch was kept for rough variation or other change, and if this was seen, or if contamination occurred, the stock cultures were discarded and replaced from frozen or freeze-dried cultures. These stock cultures were transferred no more than 10 times.

(d) Viability of cultures

A batch of strains selected at random were tested for viability. The organism was grown on horse blood agar and in cooked meat medium for 24 hours and the cultures stored in the dark at room temperature. They were tested for viability at regular intervals by inoculating on to blood agar plates and into digest broth and examining for up to 3 days for signs of bacterial growth.

(e) Examination for capsulation

Cultures grown on 0.5 per cent. maltose agar were

examined for the presence of capsules and loose slime by the wet-film India-ink method of Duguid (1951).

(f) Motility

This activity was determined by the microscopic examination of hanging-drop preparations of 24-hour-old nutrient broth cultures.

(g) Pigment formation in culture media

No special medium was used in this study. Twenty-four-hour-old nutrient agar plate cultures were stored in the dark at room temperature and examined daily for 5 days for changes in the colour of the colonies and the surrounding medium.

(h) Growth at different hydrogen ion concentrations

Finding the fact that the hydrogen ion concentrations in dextrose peptone broth appeared to affect the growth of the organisms, a group of 10 strains (RDV23, RDV28, RDV60, ERS12, ERS37, ERS47b, ERS54, ERS55, EQ2 and EQ4) selected at random was examined for their ability to grow at different hydrogen ion concentrations. One drop of a 6-hour nutrient broth culture of the organism was inoculated in Oxoid nutrient broth having the following hydrogen ion concentrations: pH 5.1, 5.6, 5.9, 6.2, 6.6, 7.0, 7.5, 8.0, 8.5, 9.0 and 9.3. The experiment was continued for 5 days.

(i) Biochemical and cultural properties

Cultures were incubated at 37°C throughout the work,

unless otherwise stated. Each strain of A.equuli investigated in the present study was subjected to the following tests:-

(i) Catalase production

(ii) oxidase activity

(iii) fermentation or oxidation of glucose

(iv) fermentative activity

(v) hydrolysis of aesculin

(vi) production of ammonia

(vii) utilization of citrates

(viii) β-galactosidase activity

(ix) hydrolysis of gelatin

(x) oxidation of gluconate

(xi) growth on MacConkey's medium

(xii) haemolysis on blood agar

(xiii) hydrolysis of hippurate

(xiv) production of hydrogen sulphide

(xv) production of indole

(xvi) KCN test

(xvii) utilization of malonate

(xviii) methylene blue reduction test

(xix) methyl red test

(xx) Vöges-Proskauer test

(xxi) reduction of nitrates

(xxii) deamination of phenylalanine

(xxiii) phosphatase test

(xxiv) hydrolysis of starch

(xxv) starch-forming properties

(xxvi) hydrolysis of urea

(i) Catalase production

The organism was grown on a slope of nutrient agar for 24 hours. One ml. of 3 per cent. hydrogen peroxide (10 vol.) was run over the growth and examined immediately and after 5 minutes for bubbles of gas which indicated a positive reaction.

(ii) Oxidase activity

On a piece of filter paper (Whatman no. 1, 3 x 4cm.) impregnated with 2 to 3 drops of 1 per cent. (w/v) aqueous tetramethyl-p-phenylenediamine dihydrochloride solution in a petri dish, a loopful of culture grown on a nutrient agar plate for 24 hours was smeared carefully across the paper with a glass rod. Production of a purple colour within 10 seconds was recorded as positive, its development in 10 to 60 seconds as delayed positive, and the absence of colouration or its still later appearance as negative (Kovács, 1956; Steel, 1961).

(iii) Fermentation or oxidation of glucose

O-F medium of Hugh and Leifson (1953) was steamed and quickly cooled immediately before use. Duplicate tubes were inoculated by stabbing with a straight wire. One of the pair of tubes was covered with a layer of sterile liquid paraffin to a depth of 0.5 to 1 cm. Tubes were incubated for up to 14 days and examined daily

for the development of a yellow colour. Fermentative activity was denoted by the production of an acid reaction throughout in both tubes and oxidative result by a yellow decolorization in the open tube only, leaving the petrolatum-sealed tube unchanged with little or no apparent growth. No change in both tubes was obtained by the non-fermenters and non-oxidizers.

(iv) Fermentative reactions

Tests were carried out in the medium recommended by Bosworth and Lovell (1944). This consisted of 1 per cent. peptone water containing 10 per cent. Lab Lemco broth, 2 per cent. bromothymol blue indicator (Cruickshank, 1972, p. 817) and 0.5 per cent. of the fermentable substrate. Organisms were inoculated with a drop of overnight nutrient broth culture in Bosworth and Lovell's sugar media containing 0.5 per cent. of the following substrates: arabinose, rhamnose, xylose, dextrose, fructose, galactose, mannose, cellobiose, lactose, maltose, melibiose, sucrose, trehalose, raffinose, inulin, dextrin, starch, glycerol, adonitol, mannitol, sorbitol, dulcitol, inositol and salicin. Incubation was continued up to 14 days and readings were made after 1, 2, 3, 4, 5, 7 and 14 day(s). The production of a full yellow colour indicated a positive reaction; incomplete colour changes were disregarded.

(v) Aesculin hydrolysis

Hydrolysis of this glycoside was determined in

cultures grown in the aesculin broth of Sneath (1956). Daily observations were made for blackening of the medium, indicating aesculin hydrolysis, for up to 7 days. With a negative result no blackening of the medium was obtained.

(vi) Ammonia production

Five-day-old peptone water cultures were tested for the presence of ammonia by the addition of Nessler's reagent (B.D.H.) as described by Wilson and Miles (1964, p. 492). A positive reaction was indicated by the development of brown colour and a negative result by a faint yellow colour.

(vii) Citrate utilization

The citrate medium of Koser (modified from Koser, 1923; Report, 1956) was used to detect the ability of the organism to utilize citrate as the sole carbon and energy source for growth and an ammonium salt as the sole source of nitrogen. Observations of growth, as shown by the development of visible turbidity, were made at intervals of one, two, three, five and seven days.

(viii) β-galactosidase activity

The peptone water medium containing o-nitrophenyl-β-d-galactopyranoside (ONPG) described by Lowe (1962) was used for this test. Positive evidence of β-galactosidase activity was indicated in cultures in this medium by the originally colourless medium becoming

yellow within 24 hours through the liberation of o-nitrophenol. A negative result was recorded when no colour change was detected after 24 hours' incubation.

(ix) Gelatin hydrolysis

Gelatin decomposition was determined by the method of Frazier (1926). Hydrolysis of gelatin was indicated by the development of clear zones around 3-day-old colonies on nutrient gelatin agar plates after flooding the surface with 5 to 10 ml. of acid mercuric chloride solution. The positive reaction was confirmed by growing the organism in nutrient gelatin for 14 days and examining for liquefaction every 2 to 3 days after cooling at $4^{\circ}C$ for 2 hours.

(x) Gluconate oxidation

The medium used for this test was the gluconate broth of Shaw and Clarke (1955). After incubation of the culture for 48 hours, 1.0 ml. of Benedict's qualitative reagent for reducing sugars was added and the bottles placed in boiling water for 10 minutes. The production of 2-keto-gluconate was indicated by the development of a yellowish brown precipitate of cuprous oxide.

(xi) Growth on MacConkey medium

MacConkey agar (Oxoid) was inoculated with a drop of overnight nutrient broth culture and examined for

the presence of growth for 3 days.

(xii) Haemolysis

The haemolytic capacity of the organism was tested on layered blood agar plates prepared with horse blood (oxalated) and sheep blood (defibrinated). The pattern of haemolysis developed on the medium was recorded after 24 hours' incubation. The degree of haemolysis was observed after further incubation for 24 hours and while storing in a dark place at room temperature.

(xiii) Hippurate hydrolysis

The organism was grown in the hippurate broth of Hare and Colebrook (1934) for 4 days. Uninoculated medium incubated for the same period of time was used as a control. To decide the exact amount of acid ferric chloride solution for the test, varying amounts of ferric chloride solution (0.2, 0.3, 0.4, 0.5 ml.) were added rapidly to 1 ml. of the control medium which was dispensed in 75 x 9.5 mm. test tube and shaken immediately. The smallest amount of acid ferric chloride solution which gave a clear solution was then added to 1 ml. of the culture. Hydrolysis of the hippurate was indicated by the formation of a heavy precipitate of ferric benzoate. With a negative test no precipitate occurred.

(xiv) Hydrogen sulphide production

Production of hydrogen sulphide was detected by

inserting a lead acetate paper strip between the cap and bottle containing nutrient broth inoculated with a drop of overnight broth culture. Blackening of this paper indicated a positive reaction. Examination of negative cultures was continued for 7 days.

(xv) Indole production

The test for indole was performed in 4-day-old Lab Lemco broth cultures. The presence of volatile indole in the medium was detected by the development of a red colour in the reagent layer after 0.5 ml. of Kovác's reagent had been added and the culture shaken.

(xvi) KCN test

The ability of the organism to grow in the presence of cyanide was tested in the KCN broth of Rogers and Taylor (1961). The medium was inoculated with one loopful of an overnight nutrient broth culture and incubated for 48 hours. The development of turbidity in the medium was taken as evidence of the test organism's tolerance of cyanide. A negative result with no sign of growth was confirmed by subculturing to the basal medium without potassium cyanide to validate the test.

(xvii) Malonate utilization

The malonate-phenylalanine medium of Shaw and Clarke (1955) was used to determine whether sodium malonate was used by the organism. A positive reaction

was denoted by the development of a deep blue colour, a negative result by the green colour of the medium remaining unchanged after 24 hours' incubation. The culture was retained for the test for phenylalanine deamination.

(xviii) Methylene blue reduction

This test was that described by Wilson and Miles (1964, p. 493). The organism was grown in nutrient broth for 24 hours. One drop of 1 per cent. aqueous methylene blue solution was added to the culture which was reincubated for one hour (see Skerman, 1969, p.779). Complete decolourization below the top half-centimetre was recorded as a positive test, green colorization as a weak positive reaction, whilst persistence of the original blue colour was seen with a negative result.

(xix) Methyl red (M.R.) test

The organism was grown for 4 days in glucose-phosphate-peptone water (Kauffmann, 1954, p. 358). The M.R. test was performed by adding 2 to 3 drops of 0.25 per cent. alcoholic solution of methyl red. A red colour developed with a positive reaction, orange colour with weak positive case and a yellow colour with a negative result.

(xx) Voges-Proskauer (V.P.) test

Acetylmethylcarbinol production from glucose, using

Barritt's (1936) modification, was tested on 4-day-old glucose-phosphate-peptone water. One ml. of a 6 per cent. alcoholic solution of α-naphthol and 0.4 ml. of 40 per cent. aqueous potassium hydroxide solution were added to 2 ml. of the culture and shaken. A positive V.P. test was indicated by the development of a strong red colour within 15 minutes.

(xxi) Nitrate reduction

The medium used for this test was that described by Kauffmann (1954, p. 357). The test to detect the reduction of nitrates to nitrites was performed after 4 days' incubation. The presence of nitrites in the nitrate broth was then indicated by the addition of 1 ml. of 0.8 per cent. solution of sulphanilic acid in 5N acetic acid and of 0.5 per cent. solution of α-naphthylamine in 5N acetic acid, a red colour developing with a positive reaction. All negative tests were confirmed by the addition of zinc dust (up to 5 mg. per ml. of culture) to reduce residual nitrate in the medium (ZoBell, 1932).

(xxii) Phenylalanine deamination

After reading the result of the malonate test, the culture was subjected to the test for the presence of phenylpyruvic acid for which purpose the method of Shaw and Clarke (1955) was adapted. The medium was acidified by the addition of 0.1 to 0.2 ml. of 0.1N

hydrochloric acid before adding 0.2 ml of 10 per cent. aqueous ferric chloride solution. A positive reaction was signified by a green colour which faded quickly and a negative result was denoted by a yellow colour.

(xxiii) Phosphatase test

A phenolphthalein phosphate agar plate was inoculated lightly to obtain well isolated colonies and after 18 hours' incubation, 0.1 ml. of ammonia solution (sp. gr. 0.880) was placed in the lid of a petri dish and the medium inverted above it. Phosphatase activity was indicated by the development of pink colonies.

(xxiv) Starch hydrolysis

A starch agar plate was inoculated with a drop of 18-hour nutrient broth culture and incubated for 5 days. Five to ten ml. of 95 per cent. ethyl alcohol was poured over the plate, a positive reaction being indicated by clear zones of hydrolysis around the area of the growth whereas milky white areas signified unchanged starch.

(xxv) Starch-forming properties

Production of iodophilic polysaccharide was tested by the method described by Phillips (1966). Organisms were grown overnight on 1 per cent. dextrose and maltose agar slopes and the cultures then flooded with Gram's iodine diluted 1 in 10. A strong positive reaction was denoted by bluish-purple coloration of the bacterial

growth and a weak positive result by reddish-brown coloration. Bacterial growth not coloured or only slightly yellow was recorded as a negative test.

(xxvi) Urea hydrolysis

The urea medium of Christensen (1946) was used to detect the presence of urease activity. Heavily inoculated slopes of the medium were incubated and readings made after 4 hours and daily for 7 days. A positive reaction was indicated by the development of a pink colour.

3. RESULTS

(a) Morphological characters

All the strains of A. equuli (including 6 NCTC strains) used in the present investigation appeared as non-motile, Gram-negative bacilli characterised by a lack of uniformity in morphology. Cells were mostly rod-shaped but there was considerable variation in length of the organism (Plate 1), and not infrequently interspersed with coccal elements or granules similar to those observed by Phillips (1966) with A. lignieresi. Often ends of the rods were more deeply stained than the central portion, giving to them a distinct bipolar appearance.

The variation in morphology was more noticeable with differences in the media on which the organism was grown. Organisms grown on nutrient agar and Loeffler's serum were mostly short bacillary or coccobacillary forms (Plates 2 and 3), but blood agar cultures of the same strain mostly showed marked pleomorphism (Plate 1). In most cases the same strain grown on glucose agar and maltose agar showed many unusual forms (Plate 4). Long rods and even filamentous forms were observed not uncommonly especially in early cultures (Plate 5). Occasionally these filamentous forms broke down into short bacillary forms and granules, thus giving an appearance of streptococcal chains (Plate 1). Large

yeast-like bodies bearing projections resembling buds and extremely big round bodies mixed with swollen rods were seen in glucose agar cultures (Plates 6 and 7). These round bodies varying in size and taking ordinary stains very poorly, looked much like transitional phase variants or L phase variants. These forms were even observed in the cultures grown on blood agar in a few cases. Swollen rods often stained faintly, giving to them an appearance of 'ghost' forms described by Phillips (1960) with A.lignieresi. Cultures grown in nutrient broth and glucose broth showed a majority of bacillary forms which were intermingled with amorphous substances. These extracellular substances were stained very poorly but were definitely Gram-negative (Plate 8).

A number of strains selected randomly were examined for the presence of capsules, but no evidence of these structures was observed in any of the cultures examined. However, considerable amounts of extracellular slime were observed in wet India ink preparations. The extracellular slime was more easily demonstrated in the stained smears of ropy sediments developed in the broth culture as faintly staining interstitial substances. This mucoid material of which the slime is composed makes broth cultures viscid and colonies sticky and is very difficult to remove from an agar surface.

Plate 1 24-hour blood agar culture of A. equuli (strain ERS12) showing pleomorphism of the organism. Gram. X2500.

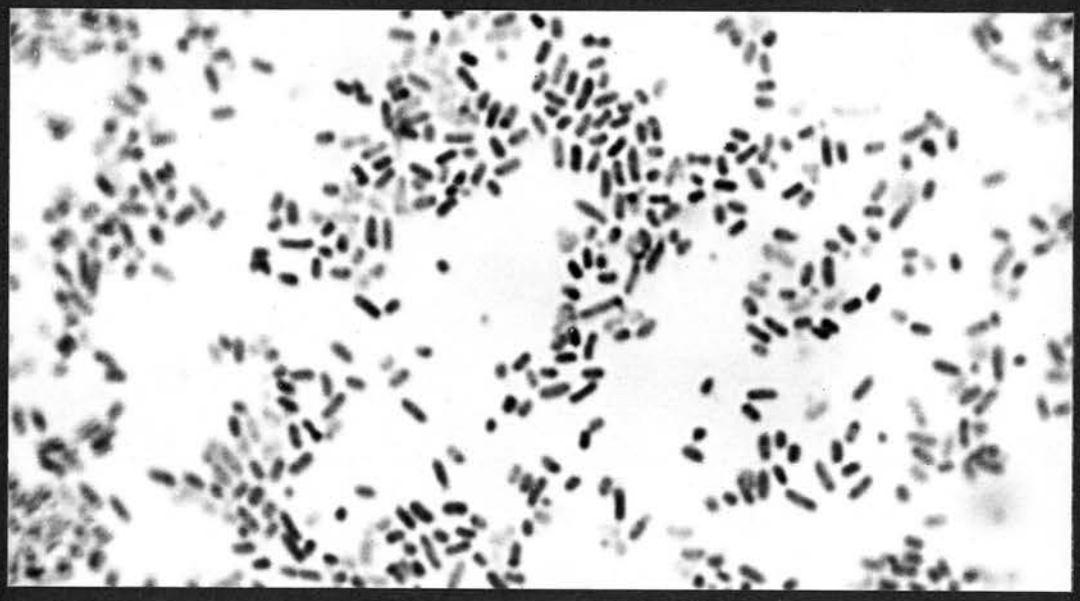

Plate 2 24-hour nutrient agar culture of A. equuli (strain ERS12) showing mostly bacillary and coccobacillary forms. Gram. X2500.

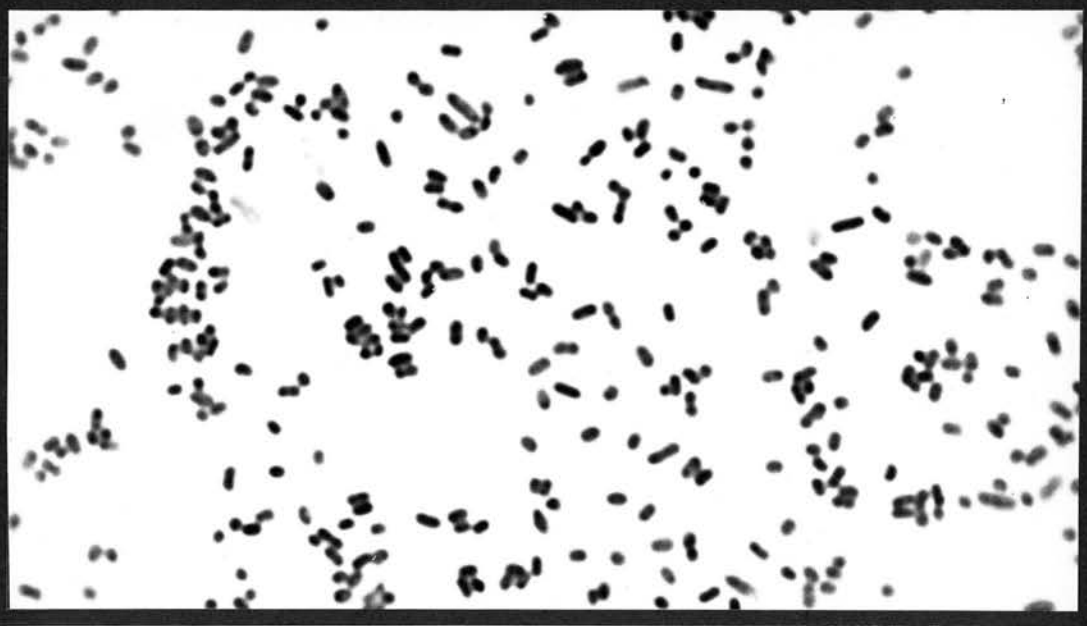

Plate 3 24-hour Loeffler's serum slope culture of A. equuli (strain ERS12) showing mostly coccobacillary and bacillary forms. Gram. X2500.

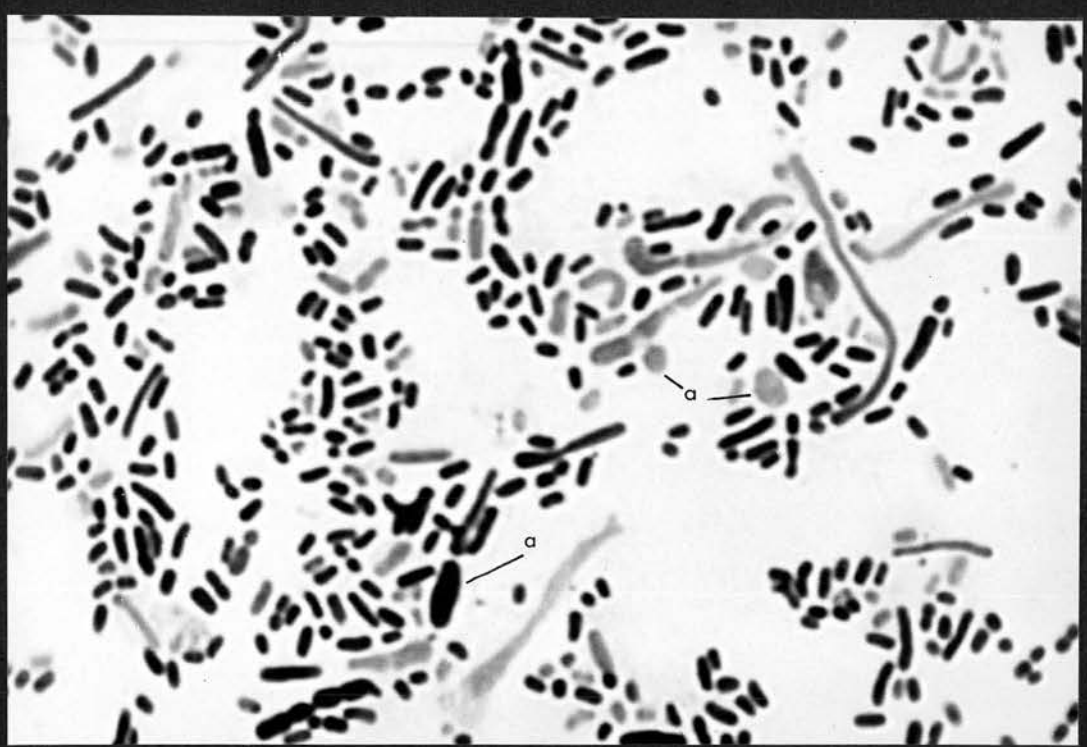

Plate 4 24-hour glucose agar culture of A. equuli (strain ERS12) showing unusual forms(a). Gram. X2500.

Plate 5 24-hour blood agar culture of A. equuli (strain NCTC8644) showing filamentous forms. Gram. X1500.

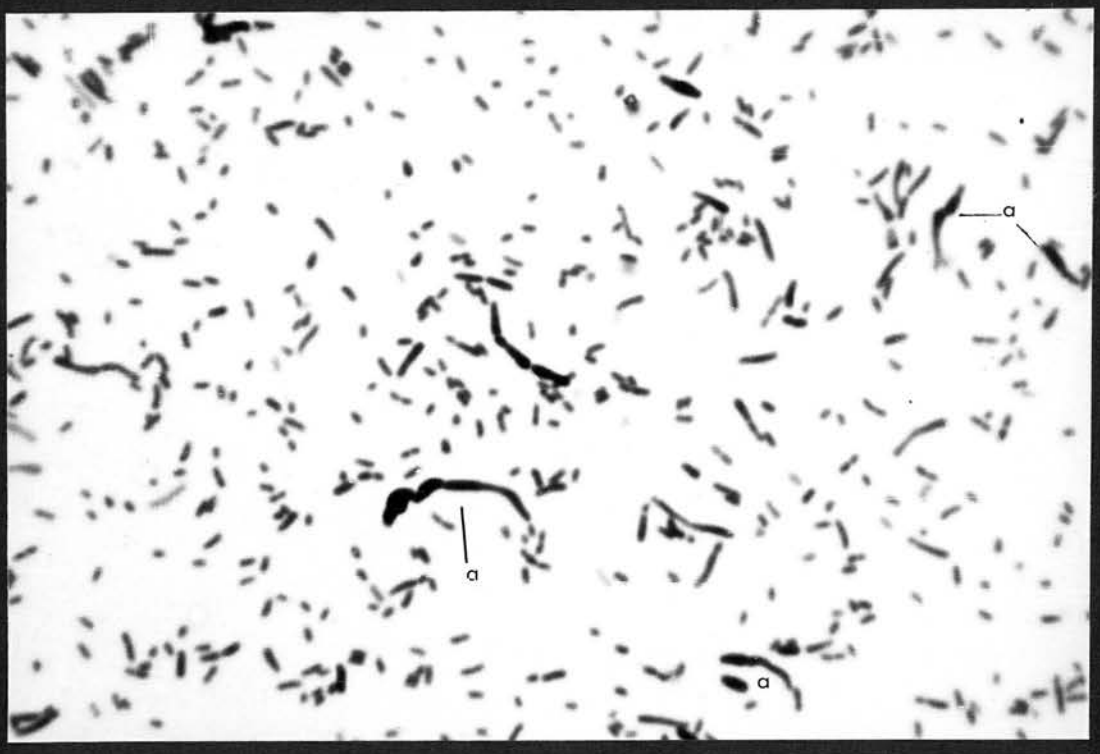

Plate 6 24-hour glucose agar culture of A. equuli (strain NCTC8644) showing unusual forms(a). Gram. X1500.

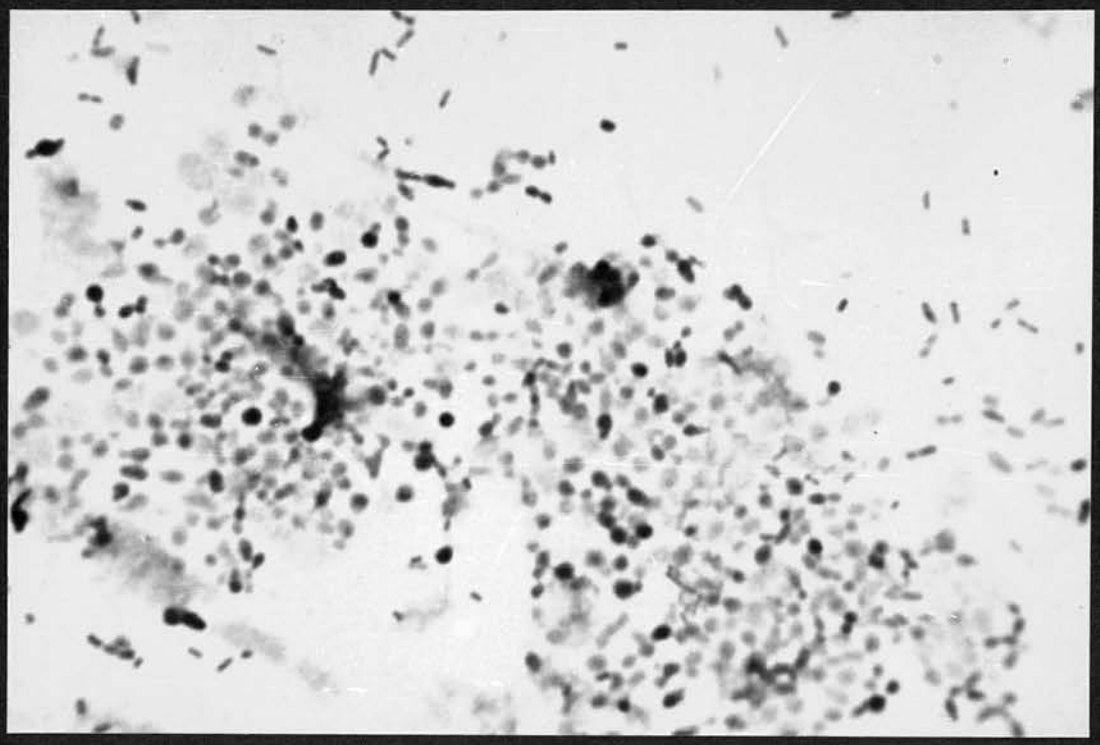

Plate 7 24-hour glucose agar culture of A. equuli (strain EQ3) showing round bodies. Gram. X1500.

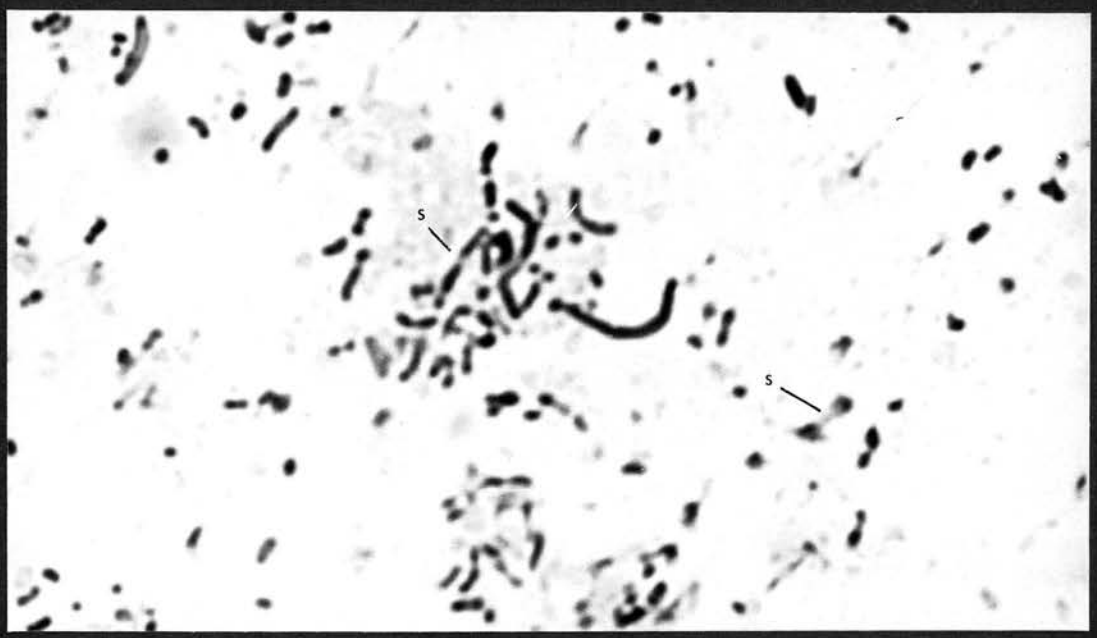

Plate 8 48-hour blood agar culture of A. equuli (strain NCTC8644) showing evidence of extracellular slime(s). Gram. X2500.

Table 15 Summary of biochemical and fermentative characteristics of *Actinobacillus equuli* reported by previous workers

	Ashford & Shirlaw(1962)	Baker(1972)	Carter et al.(1971)	Cottew & Francis(1954)	Cowan & Steel (1970)	Dimock et al.(1928)	Edwards(1931)	Frederiksen(1973)	Haupt(1934)	Hirato(1939)	Hutchinson(1956)	Jones & Simmons(1971)	Magnusson(1931)	Mair et al.(1974)	Meixner(1931)	Meyer(1910)	Moon et al.(1969)	Mráz et al.(1968)	Neter(1942)	Osbaldiston & Walker(1972)	Phillips(1966)	Ross et al.(1972)	Sakazaki & Watanabe(1956)	Snyder(1925)	Ubertini(1933)	Vallée et al.(1963)	Wetmore et al.(1963)	Windsor(1973)	Zimmermann(1964)
No. of strains examined	1	1	1	31	.	.	40	6	6	27	1	1	5	7	5	1	2	3	.	3	8	1	10	.	22	11	5	2	.
Catalase	.	.	-	.	d	.	.	-	.	.	.	+	.	-	.	.	.	+	.	-	d	-	.	.	.	.	.	-	.
Oxidase	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	-	.
Aesculin hydrolysis	.	.	.	.	.	.	.	-	.	.	.	.	.	-	.	.	.	.	.	-	.	-	.	.	.	.	.	.	.
Ammonia production	.	.	.	.	.	.	±	.	+	.	.	.	.	.	.	.	.	-	.	.	+	.	.	.	.	.	.	.	.
Citrate utilization	-	-	-	.	-	.	-	.	-	.	-	.	.	-	.	.	-	-	.	-	.	-	-	.	.	.	.	+	.
Gelatin liquefaction	.	-	.	.	-	-	-	d	-	.	-	.	-	-	.	+	.	-	-	-	.	-	-	-	.	.	-	-	.
Gluconate oxidation	.	.	.	.	-	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H_2S production	-	.	-	.	-	.	.	-	+	+	.	.	-	-	.	-	+	+	.	+	-	+	-	.	+	-	-	-	+
Indole production	-	-	-	-	-	-	-	-	-	.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	.	-	-
KCN test	.	.	.	.	-	.	.	-	.	.	.	.	.	-	-	.	.	.	.	+	.	.	.	.	.	.	.	.	.
Growth on MacConkey	-	.	.	.	d	.	.	.	.	.	.	+	.	+	.	.	.	.	.	.	d	.	.	.	.	.	+	+	.
Malonate utilization	.	.	.	.	-	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.
Methylene blue redn.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	-	.	.	.	.	.	.	.	.
Methyl red test	-	.	-	.	-	.	-	.	.	.	-	.	.	-	.	.	-	-	.	+	-	-	-	.	.	-	.	-	.
Voges-Proskauer test	-	.	-	.	-	.	-	-	-	.	-	.	.	-	.	.	-	-	.	-	-	-	-	.	.	-	.	-	.
ONPG	.	.	.	.	+	.	.	+	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Nitrate reduction	+	.	+	.	.	.	+	+	+	.	+	+	.	+	.	.	+	+	+	+	+	+	+	.	.	+	+	+	+
Phenylalanine	.	.	.	.	.	.	.	-	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Starch from glucose	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	±	.	.	.	.	.	.	.	.
Starch from maltose	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	±	.	.	.	.	.	.	.	.
Urea hydrolysis	+	.	+	.	+	.	.	+	.	.	.	+	.	+	.	.	-	+	.	+	+	+	+	.	.	+	+	+	.
Haemolysis of sheep rbc	-	-	+	d	-	-	.	-	.	.	.	-	-	-	.	-	-	+	.	+	.	.	.	.	+	.	-	-	-
Gas from glucose	-	-	-	-	-	.	-	.	-	.	-	-	-	-	-	-	-	-	.	-	-	-	.	-	-	.	.	-	.
Fermentation of																													
Arabinose	-14	.	-7	.	.	-	-	-	-	d	-	.	-	-	+	.	.	+	.	.	+	-	.	-	+	.	.	.	-10
Rhamnose	-14	.	-7	-	.	.	-	-	.	-	-	.	-	-	.	.	.	-28	-	.	d	-	.	-	+	.	.	.	-10
Xylose	+	+	-7	+	.	.	+	+	+	+	+	.	+	+	.	.	.	+	+	+	+	+	+	+	+	+	.	.	.
Glucose	+	+	+2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	.	.	+	.	.	.	.	+	+	.	.	+	+	.	+	.	+	+	.	+	.	.	+	+	+	.	.	.
Galactose	+tr	+	.	d	.	+	+	+	+	+	+	.	+	+	.	+	.	+	+	.	+	+	.	.	+	+	.	.	.
Mannose	+	.	.	+	.	.	.	+	.	+	+	.	+	+	.	+	.	+	.	.	+	+	.	.	+	.	.	.	.
Cellobiose	.	.	.	.	.	.	.	-	.	.	.	.	.	-	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.
Lactose	+	+	+2	+	+	+	+	+	+	+	+	+	+	+	+	.	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	+tr	+	+2	+	.	+	+	+	+	+	+	+	+	+	.	+	+	+	+	-	+	+	+	+	+	+	+	.	.
Melibiose	.	.	.	.	.	.	.	+	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.
Sucrose	+tr	+	+2	+	+	+	+	+	+	+	+	+	+	+	+	.	+	+	+	+	+	+	+	+	+	+	+	+	.
Trehalose	.	.	+2	d	.	.	.	.	.	+	+	+	.	+	.	.	.	+	.	.	+	-	+	.	.	.	.	.	.
Raffinose	+	+	+2	+	.	+	+	+	+	+	+	.	.	+	.	.	.	+	+	.	+	+	.	.	+	.	-	.	.
Inulin	-14	.	.	-	.	.	-	.	-	-	.	.	-	-	.	.	.	-28	.	.	-14	-	.	.	-	+	.	.	.
Dextrin	+	.	+2	d	.	.	+	.	-	+	.	.	-	d	-	.	.	(+)	.	.	+	.	.	-	+	+	.	.	.
Starch	.	.	.	+	.	.	-	.	-	d	-	.	.	.	.	.	.	(+)	.	.	.	.	.	.	.	+	.	.	.
Glycerol	-14	.	-7	d	-	.	(+)	.	-	d	.	.	-	d	-	.	.	(+)	.	.	d	.	.	.	-	-	.	.	.
Adonitol	+	.	.	-	.	-	-	-	-	.	.	.	-	-	.	.	.	-28	.	.	.	-	-	.	-	.	.	.	.
Mannitol	+tr	+	-7	-	+	+	+	+	+	d	+	+	+	+	+	+	+	+	+	-	+	+	d	.	d	d	+	+	+
Sorbitol	+tr	.	-7	-	-	.	-	-	-	-	-	.	-	-	.	.	-	-28	-	.	d	+	-	-	-	.	d	.	.
Dulcitol	-14	.	.	-	-	-	-	-	-	-	-	.	-	-	-	+	-	-28	-	.	-14	-	-	-	-	-	.	-	-10
Inositol	-14	.	-7	-	.	.	.	-	.	-	-	.	.	-	.	.	-	-28	.	.	-14	-	-	-	.	.	.	.	.
Salicin	-14	.	+2	+	.	.	-	-	d	d	-	.	-	-	.	.	-	+	.	.	-14	-	d	+	d	.	.	.	.

+ = More than 80 per cent. strains positive
d = 21 to 79 per cent. strains positive
- = More than 80 per cent. strains negative
. = No information

+2, +14 = More than 80 per cent. strains positive within 2 days, 14 days
(+) = Delayed reaction
± = Weak positive reaction
+tr = Trace reaction

TABLE 16 BIOCHEMICAL AND FERMENTATIVE REACTIONS OF 138 STRAINS OF A. EQUULI FROM VARIOUS SOURCES

	Catalase positive	Catalase negative	Oxidase positive	Oxidase late positive	Oxidase negative	Aesculin positive	Aesculin negative	Haemolysis(sheep rbc) positive	Haemolysis(sheep rbc) negative	Hippurate positive	Hippurate negative	H_2S positive	H_2S negative	Methylene blue positive	Methylene blue weak positive	Methylene blue negative	Starch from glucose positive	Starch from glucose weak positive	Starch from glucose negative
Catalase positive	40																		
Catalase negative		98																	
Oxidase positive	5	15	20																
Oxidase late positive	25	56		81															
Oxidase negative	10	27			37														
Aesculin positive	10	38	5	25	18	48													
Aesculin negative	30	60	15	56	19		90												
Haemolysis of sheep rbc positive	12	40	12	24	16	47	5	52											
Haemolysis of sheep rbc negative	28	58	8	57	21	1	85		86										
Hippurate positive	32	92	14	74	33	43	81	46	78	124									
Hippurate negative	8	6	6	3	4	5	9	6	8		14								
H_2S positive	14	20	11	14	9	15	19	18	16	28	6	34							
H_2S negative	26	78	9	67	28	33	71	34	70	96	8		104						
Methylene blue reduction positive	23	58	14	48	19	24	57	27	55	74	7	22	59	81					
Methylene blue reduction weak positive	6	19	3	15	7	12	17	8	17	22	3	4	21		25				
Methylene blue reduction negative	11	21	3	18	11	12	16	17	16	28	4	8	24			32			
Starch from glucose positive	1	0	0	1	0	0	1	1	0	0	1	1	0	1	0	0	1		
Starch from glucose weak positive	4	2	2	4	0	2	4	2	4	4	2	3	3	3	2	1		6	
Starch from glucose negative	35	96	18	76	37	46	85	49	82	120	11	30	101	77	23	31			131
Starch from maltose positive	4	8	3	7	2	3	9	4	8	9	3	3	9	7	2	3	1	0	11
Starch from maltose weak positive	14	14	3	16	9	4	24	4	24	24	4	8	20	19	6	3	0	5	23
Starch from maltose negative	12	86	14	58	26	41	57	44	54	91	7	23	75	55	17	26	0	1	97
Arabinose positive	8	7	1	13	1	6	9	6	9	13	2	3	12	5	4	6	0	1	14
Arabinose late positive	10	23	7	17	9	13	20	14	19	31	2	7	26	21	6	6	0	0	33
Arabinose negative	22	68	12	51	27	29	61	32	58	80	10	24	66	55	15	20	1	5	84
Galactose positive	32	72	15	61	28	45	59	48	56	93	11	29	75	63	16	25	1	5	98
Galactose late positive	7	24	4	20	7	3	28	4	27	28	3	4	27	16	8	7	0	1	30
Galactose negative	1	2	1	0	2	0	3	0	3	3	0	1	2	2	1	0	0	0	3
Mannose positive	19	24	10	27	6	16	27	19	24	33	10	12	31	9	8	26	1	5	37
Mannose late positive	20	64	9	49	26	28	56	29	55	81	3	20	64	67	12	5	0	1	83
Mannose negative	1	10	1	5	5	4	7	4	7	10	1	2	9	5	5	1	0	0	11
Cellobiose positive	9	17	4	16	6	26	0	26	0	23	3	6	20	12	2	12	0	2	24
Cellobiose late positive	1	17	5	6	7	18	0	18	0	16	2	8	10	9	6	3	0	0	18
Cellobiose negative	30	64	11	59	24	4	90	8	86	85	9	20	74	60	17	17	1	4	89
Lactose positive	32	96	16	78	34	48	80	52	76	119	9	29	99	74	23	31	1	4	123
Lactose late positive	8	2	4	3	3	0	10	0	10	5	5	5	5	7	2	1	0	2	8
Melibiose positive	25	70	14	58	23	35	60	37	58	91	4	21	74	56	17	22	0	2	93
Melibiose late positive	9	28	2	21	14	13	24	14	23	33	4	7	30	21	7	9	0	2	35
Melibiose negative	6	0	4	2	0	0	6	1	5	0	6	6	0	4	1	1	1	2	3
Trehalose positive	33	90	16	74	33	48	75	50	73	115	8	26	97	73	21	29	0	4	119
Trehalose late positive	1	7	0	5	3	0	8	1	7	8	0	2	6	3	3	2	0	0	8
Trehalose negative	6	1	4	2	1	0	7	1	6	1	6	6	1	5	1	1	1	2	4
Raffinose positive	30	93	16	71	36	47	76	50	73	116	7	25	98	72	21	20	0	4	119
Raffinose late positive	6	5	0	10	1	1	10	2	9	8	3	5	6	7	3	1	1	0	10
Raffinose negative	4	0	4	0	0	0	4	0	4	0	4	4	0	2	1	1	0	2	2
Glycerol positive	5	7	2	5	5	4	8	5	7	9	3	4	8	8	3	1	1	1	10
Glycerol late positive	34	80	17	69	28	42	72	45	69	104	10	29	85	67	18	29	0	5	109
Glycerol negative	1	11	1	7	4	2	10	2	10	11	1	1	11	6	4	2	0	0	12
Mannitol positive	24	54	9	49	20	8	70	9	69	72	6	18	60	52	14	12	1	4	73
Mannitol late positive	4	12	1	10	5	0	16	0	16	13	3	2	14	7	4	5	0	0	16
Mannitol negative	12	32	10	22	12	40	4	43	1	39	5	14	30	22	7	15	0	2	42
Sorbitol positive	2	21	3	15	5	7	16	7	16	23	0	4	19	17	5	1	0	1	22
Sorbitol negative	38	77	17	66	32	41	74	45	70	101	14	30	85	64	20	31	1	5	109
Salicin positive	10	34	9	22	13	44	0	44	0	39	5	14	30	21	8	15	0	2	42
Salicin late positive	0	3	1	1	1	2	1	3	0	3	0	1	2	2	0	1	0	0	3
Salicin negative	30	61	10	58	23	2	89	5	86	82	9	19	72	58	17	16	1	4	86

	Starch from maltose positive	Starch from maltose weak positive	Starch from maltose negative	Arabinose positive	Arabinose late positive	Arabinose negative	Galactose positive	Galactose late positive	Galactose negative	Mannose positive	Mannose late positive	Mannose negative	Cellobiose positive	Cellobiose late positive	Cellobiose negative	Lactose positive	Lactose late positive
Starch from maltose positive	12																
Starch from maltose weak positive		28															
Starch from maltose negative			98														
Arabinose positive	1	1	13	15													
Arabinose late positive	1	5	27		33												
Arabinose negative	10	22	58			90											
Galactose positive	10	21	73	14	26	64	104										
Galactose late positive	2	7	22	1	7	23		31									
Galactose negative	0	0	3	0	0	3			3								
Mannose positive	5	8	30	10	13	20	41	2	0	43							
Mannose late positive	6	20	58	4	18	62	57	26	1		84						
Mannose negative	1	0	10	1	2	8	6	3	2			11					
Cellobiose positive	3	4	19	5	5	16	25	1	0	12	14	0	26				
Cellobiose late positive	0	0	18	1	5	12	18	0	0	4	10	4		18			
Cellobiose negative	9	24	61	9	23	62	61	30	3	27	60	7			94		
Lactose positive	10	26	92	14	31	83	98	28	2	38	82	8	26	18	84	128	
Lactose late positive	2	2	6	1	2	7	6	3	1	5	2	3	0	0	10		10
Melibiose positive	7	20	68	14	23	58	71	21	3	26	64	5	20	12	63	92	3
Melibiose late positive	2	5	30	1	10	26	28	9	0	11	20	6	6	6	25	35	2
Melibiose negative	3	3	0	0	0	6	5	1	0	6	0	0	0	0	6	1	5
Trehalose positive	8	24	91	15	33	75	94	27	2	34	80	9	26	18	79	118	5
Trehalose late positive	1	1	6	0	0	8	4	3	1	2	4	2	0	0	8	8	0
Trehalose negative	3	3	1	0	0	7	6	1	0	7	0	0	0	0	7	2	5
Raffinose positive	8	25	90	14	32	77	95	26	2	37	79	7	26	17	80	120	3
Raffinose late positive	2	1	8	1	1	9	6	4	1	2	5	4	0	1	10	8	3
Raffinose negative	2	2	0	0	0	4	3	1	0	4	0	0	0	0	4	0	4
Glycerol positive	2	6	4	1	2	9	12	0	0	6	6	0	3	1	8	10	2
Glycerol late positive	9	21	84	13	29	71	86	27	1	35	71	8	23	15	76	107	7
Glycerol negative	1	1	10	1	1	10	6	4	2	2	7	3	0	2	10	11	1
Mannitol positive	8	22	48	8	12	58	55	21	2	26	47	5	3	2	73	68	10
Mannitol late positive	1	3	12	1	6	9	6	9	1	0	14	2	0	0	16	16	0
Mannitol negative	3	3	38	6	15	23	43	1	0	17	23	4	23	16	5	44	0
Sorbitol positive	1	1	21	1	6	16	14	9	0	4	17	2	1	2	20	23	0
Sorbitol negative	11	27	77	14	27	74	90	22	3	39	67	9	25	16	74	105	10
Salicin positive	3	4	37	6	10	28	43	1	0	16	24	4	26	18	0	44	0
Salicin late positive	0	0	3	0	2	1	1	2	0	0	3	0	0	0	3	3	0
Salicin negative	9	24	58	9	21	61	60	28	3	27	57	7	0	0	91	81	10

	Melibiose positive	Melibiose late positive	Melibiose negative	Trehalose positive	Trehalose late positive	Trehalose negative	Raffinose positive	Raffinose late positive	Raffinose negative	Glycerol positive	Glycerol late positive	Glycerol negative	Mannitol positive	Mannitol late positive	Mannitol negative	Sorbitol positive	Sorbitol negative	Salicin positive	Salicin late positive	Salicin negative
Melibiose positive	95																			
Melibiose late positive		37																		
Melibiose negative			6																	
Trehalose positive	88	35	0	123																
Trehalose late positive	7	1	0		8															
Trehalose negative	0	1	6			7														
Raffinose positive	90	33	0	116	6	1	123													
Raffinose late positive	5	4	2	7	2	2		11												
Raffinose negative	0	0	4	0	0	4			4											
Glycerol positive	8	1	3	8	0	4	9	1	2	12										
Glycerol late positive	75	36	3	105	6	3	103	9	2		114									
Glycerol negative	12	0	0	10	2	0	11	1	0			12								
Mannitol positive	50	22	6	65	6	7	65	9	4	8	62	8	78							
Mannitol late positive	12	4	0	15	1	0	15	1	0	0	14	2		16						
Mannitol negative	33	11	0	43	1	0	43	1	0	4	38	2			44					
Sorbitol positive	14	9	0	20	2	1	19	4	0	1	20	2	20	2	1	23				
Sorbitol negative	81	28	6	103	6	6	104	7	4	11	94	10	58	14	43		115			
Salicin positive	32	12	0	44	0	0	43	1	0	4	38	2	4	0	40	3	41	44		
Salicin late positive	1	2	0	3	0	0	3	0	0	0	3	0	3	0	0	3	0		3	
Salicin negative	62	23	6	76	8	7	77	10	4	8	73	10	71	16	4	17	74			91

(b) Cultural characters

(i) Cultures on solid media

All organisms examined grew readily on the ordinary culture media. The growth on blood agar was profuse and often discoloured the medium greenish brown on prolonged cultivation. In some cases a zone of haemolysis developed around the colony. The most characteristic feature of the organisms grown on agar media was that they were viscous and difficult to remove completely from the surface. On hooking them with an inoculating loop, the colonies were found to be ropy and pulled out like elastic in most cases. The number of strains giving sticky colonies and ropy broth sediment are shown in Table 2, from which it can be seen that no less than 89.9 per cent. of the strains studied developed sticky colonies on agar plates. The organisms grew in either of two forms, the 'rough' or the 'smooth', these adjectives being descriptive of the colonies developed. The rough forms of the colonies on agar media were raised, opaque, with an irregular surface, mostly wrinkled, dry and extremely tenacious. The edges of the colonies were undulate. The colonies on blood agar attained a size of 1 to 2 mm. in diameter within 24 hours and gradually increased up to 3 to 4 mm. in diameter with further incubation. With increasing age a clear, smooth border zone was developed from the opaque centre. The rough form of

Table 2

The ability of 138 strains of *A. equuli* to produce sticky colonies or broth deposit

	Agar colony		Broth deposit	
	Sticky	Not sticky	Ropy	No sediment
Equine strains:				
pathological material	16	1	15	2
normal animal	94	6	98	2
uncertain origin	4	1	3	2
Porcine strains	5	5	5	5
NCTC strains	5	1	5	1
Total	124(89.9%)	14(10.1%)	126(91.3%)	12(8.7%)

the colony appeared to be unstable and easily tended towards the smooth form on continued subcultivation. The smooth forms of the colonies on agar media after 24 hours incubation were entire edged, smooth surfaced, greyish white in colour with whitish opaque centres. The colonies were flat or slightly raised and were 1 to 2 mm. in diameter after 24 hours cultivation. Prolonged incubation showed a considerable increase in the size of colonies with the projection of a clear border zone from the greyish white, translucent centre, thus often giving an appearance like a fried egg.

In a few cases a 'smooth mucoid' form characterised by its moist and glistening appearance, greyish white in colour, was found. These colonies were much like those of the mucoid forms of Pasteurella multocida.

(ii) Cultures in liquid media

The first growth of the organisms to become apparent in nutrient broth was a collection of small masses of bacteria at the bottom of the culture bottle. After 12 hours, nutrient broth was slightly turbid and a distinct sediment was formed in most cases. A characteristic growth appearing in broth within 24 hours was the formation of a sediment mostly of a ropy nature. In 91.3 per cent. of strains examined, the characteristic bacterial sediment in nutrient broth culture was observed within 24 hours (see Table 2). The deposit which

settled at the bottom of the bottle was rather tenacious and of mucous consistency and by shaking the bottle gently the whole mass rose up as a slimy cone into the liquid above and remained suspended for some time because the whole medium was very viscous. The growth in peptone water was rather poorer than that in ordinary broth, although the characteristic ropy sediment observed in broth culture was also seen. In cooked meat medium a slight superficial membrane of uneven thickness was often present floating on the surface of medium and the side of the bottle especially was covered with a deposit of bacteria.

The fact that unusual forms of the organisms were noted especially with the medium containing dextrose, prompted a test to be made on the effect of dextrose on the growth of the organism. A batch of 18 strains of A.equuli selected randomly was tested for the ability to grow in peptone broth containing different concentrations of dextrose. One drop of 6-hour peptone broth cultures of the organism was inoculated into each of four peptone broths containing 1, 2, 5 and 10 per cent. of dextrose respectively. The experiment was continued for 5 days. In all cases no growth occurred in either 5 per cent. or 10 per cent. dextrose peptone broth while good growth was obtained in the broth having dextrose concentrations of 1 and 2 per cent. There were no morphological differences between the organisms grown

in 1 per cent. dextrose broth and those cultivated in 2 per cent. dextrose medium, but the viscosity of broth appeared to become more marked with the increase of the dextrose concentration of the medium. It was found that the hydrogen ion concentrations of the 5 and 10 per cent. dextrose broths in which the organisms had failed to grow were not within the expected range of pH 7.2 to 7.4. The media had been autoclaved at 121°C for 15 minutes. To test for the final hydrogen ion concentrations of dextrose medium after autoclaving, 3 x 10 ml. amounts of each required percentage of dextrose peptone broth were made at the initial reaction of pH 7.7 and then autoclaved at 121°C for 15 minutes in the same way as 1 per cent. dextrose broth for general use was done. The final pH of the control medium (peptone broth) remained unchanged but considerable variations in hydrogen ion concentrations of dextrose containing media were noted. The final pH of 1, 2, 5 and 10 per cent. dextrose peptone broth were 7.3, 7.05, 6.6 and 6.2 respectively. The higher the concentration of dextrose in medium, the greater was the variation in hydrogen ion concentration observed. Another batch of dextrose peptone broth in which sterile dextrose solution was added aseptically to give the required concentrations was used to test for the ability of the organism to grow. In all cases the growth occurred in both 5 and 10 per cent. dextrose peptone broth. The medium became

extremely viscous. When an inoculating loop was put into the medium and then withdrawn slowly, the medium adhering to it was drawn out like an elastic band. Often the ropy sediment was mixed with granular material.

(c) Growth at different hydrogen ion concentrations

The organisms appeared to be sensitive to acid, but more tolerant of alkaline conditions. No growth occurred in the broths having reactions of pH 5.1, 5.6, 5.9 and 9.3. Growth was delayed and poor in the broths having reactions of pH 6.2 and 8.5, while the maximum growth was observed in the broths having reactions of pH 7.5 and 8.0. Good growth was obtained at pH 6.6 and 7.0. Six of 10 strains were able to grow in the broth having a reaction of pH 9.0 even though the growth was markedly poor and delayed.

(d) Viability of A. equuli on ordinary culture media

Thirty strains of A. equuli selected randomly from the 138 strains studied were examined for their viability on horse blood agar and in cooked meat medium in order to determine the maximum duration of storage of cultures at room temperature on or in those media for day-to-day working purposes. The results obtained are given in Tables 3 and 4. On blood agar most of the cultures did not remain viable for more than 7 days even if kept in the dark at room temperature, and after 14 days no organisms at all remained alive (Table 3).

Table 3

Survival of 30 strains of Actinobacillus equuli on blood agar plate at room temperature

Duration of storage (days)	No. of strains dead	Cumulative total of dead strains	Percentage of viability
3	0	0	100
5	0	0	100
7	16	16	47
9	6	22	27
11	5	27	10
14	3	30	0

Table 4

Survival of 30 strains of Actinobacillus equuli in cooked meat medium at room temperature

Duration of storage (days)	No. of strains dead	Cumulative total of dead strains	Percentage of viability
14	0	0	100
17	0	0	100
21	0	0	100
24	1	1	97
28	11	12	60
31	12	24	20
35	5	29	3
42	1	30	0

Cultures in cooked meat medium, however, remained alive for 3 to 4 weeks (Table 4). After 6 weeks no organism remained viable in this medium. In practice, therefore, the organism was propagated at three-weekly intervals in cooked meat medium and kept at room temperature in a dark place. All through the work it proved satisfactory to keep the stock cultures in cooked meat medium but they were replaced, in any event, after about 10 transfers.

(e) Biochemical characters

The 138 strains studied fermented dextrose without the production of gas. As many as 97 per cent. of the strains fermented dextrose within 24 hours indicating that the fermentative activity of A. equuli on dextrose is very prompt. Moreover, four strains (ERS33, ERS44, ERS54 and RDV44) which failed to ferment dextrose within 24 hours did so within 2 days. All strains fermented fructose, lactose, sucrose and dextrin mostly within 24 hours. The rate of prompt fermentation (within 24 hours) with these substrates was 94, 93, 94 and 78 per cent., respectively. Fermentation of xylose and maltose was observed in all strains with the exception of one in each case. The only one strain which failed to ferment xylose within 14 days was EQ6 which was isolated from a case of endocarditis in a 30-day-old piglet and the strain which failed to ferment maltose was ERS5 of uncertain origin. None of the strains

fermented rhamnose, inulin, adonitol, dulcitol or inositol. With the remaining substrates used in the present study differences in the reactions between strains were observed. Especially with arabinose, cellobiose, mannitol and salicin discrepancies in the fermentation test between strains were noted. The differences for the 132 strains studied and the 6 National Collection of Type Cultures strains are summarized in Table 5 and individually presented in Appendix A. More detailed figures of the strains giving various patterns of fermentation are given in Table 16 (p.108). As shown in Table 5, there was no marked disagreement in most of the fermentation reactions between the 132 strains examined and the 6 NCTC strains. However, none of the NCTC strains fermented cellobiose or salicin although 44 and 47 per cent. respectively of the other strains did attack these two substrates. Mannitol was fermented by all NCTC strains but 33 per cent. of the other group failed to attack this substrate.

Arabinose was fermented by 48(35%) of 138 strains studied, but this occurred mostly later than the first 24 hours of incubation, two-thirds of positive reactions appearing at 2 to 5 days. One hundred and thirty-five strains (98%) attacked galactose with the range of 1 to 5 days but the majority of strains (77%) which utilized the substrate did so within 24 hours. Mannose

Table 5

Fermentation reactions of 138 strains of A. equuli

Substrate	Strains from various sources		NCTC strains	
	Positive	Negative	Positive	Negative
Arabinose	47(32*)	85	1(1)	5
Xylose	131(10)	1	6	0
Dextrose	132(4)	0	6	0
Fructose	132(9)	0	6	0
Galactose	130(28)	2	5(3)	1
Mannose	122(81)	10	5(3)	1
Cellobiose	44(18)	88	0	6
Lactose	132(10)	0	6	0
Maltose	131(18)	1	6(2)	0
Melibiose	126(35)	6	6(2)	0
Sucrose	132(8)	0	6	0
Trehalose	125(8)	7	6	0
Raffinose	128(9)	4	6(2)	0
Dextrin	132(28)	0	6(3)	0
Starch	24(24)	108	1(1)	5
Glycerol	122(110)	10	4(4)	2
Mannitol	88(14)	44	6(2)	0
Sorbitol	23(3)	109	0	6
Salicin	47(3)	85	0	6

* Figures in the parentheses indicate number of strains showing late fermentation(negative at 24 hours; positive within 14 days).

was fermented by 127 (92%) of the strains and of these sixty-six per cent. did so between the 2nd and 5th days. Cellobiose was fermented by 44 strains (32%), of which 26 strains (59%) did so promptly, while salicin was utilized by 47 strains (34%) within 24 hours with the exception of 3 which did so late by the 7th day. As many as 132 strains (96%) fermented melibiose mostly with prompt reaction but six failed to attack the disaccharide within 14 days. Similarly, trehalose and raffinose were attacked by the majority of strains with the rates of positive reaction being 95 and 97 per cent., respectively, and mostly with prompt reactions. Only 4 strains failed to ferment raffinose and 7 strains did not attack trehalose after the 2-weeks period of incubation. Starch was fermented by only 25 strains (18%) while glycerol was utilized by 126 strains (91%). Of those strains which fermented starch and glycerol, most did so late, the average times for fermentation of these two substrates being 6 days and 3 days, respectively. Variable results were obtained with the substrates of mannitol and sorbitol which were fermented by 94 (68%) and 23 (17%) strains, respectively. The majority of strains fermenting mannitol did so within 24 hours with the exception of 16 strains which attacked the alcohol between 2 and 3 days. Sorbitol was fermented by a relatively small number of strains but the prompt fermentation of this substrate was obtained from 20 (87%)

of the 23 strains which showed a positive reaction.

Neither ammonia nor indole was produced by any of the strains of A. equuli examined in the present work. All strains failed to utilize citrate as the sole carbon and energy source for growth but they all reduced nitrate to nitrite with the exception of one. The methyl red test was negative in all cases but 5 strains reacted positively in the Vöges-Proskauer test, the others showing no sign of the presence of acetyl methyl-carbinol in the culture medium. No organism showed positive reactions in either the malonate or phenylalanine tests. Phosphatase and urease activities were positive in all strains tested. No evidence of the hydrolysis of gelatin was detected in any case during 14 days of examination for the presence of liquefaction of nutrient gelatin but, with the method of Frazier (1926), two cases of a positive reaction were observed. All strains were proved to be able to induce β-galactosidase but failed to convert gluconate to 2-keto gluconate.

With the remaining biochemical tests employed there were considerable variations in the reactions between strains and these are summarised in Table 6 and presented individually in Appendix A. More detailed figures of the strains giving various patterns of biochemical properties are given alongside the results of their fermentative characters in Table 16 (p. 108). The presence of the enzyme catalase was observed in 40

Table 6

Biochemical reactions of 138 strains of A. equuli

Characters	Strains from various sources		NCTC strains	
	Positive	Negative	Positive	Negative
Catalase	39	93	1	5
Oxidase	96(77*)	36	5(4)	1
Aesculin hydrolysis	48	84	0	6
Hydrolysis of gelatin(plate)	1	131	1	5
Hippurate	119	13	5	1
H_2S production	32	100	2	4
KCN	1	131	0	6
Growth on MacConkey medium	131	1	6	0
Methylene blue reduction	102(25)	30	4	2
Nitrate reduction	131	1	6	0
Starch from glucose	7(6)	125	0	6
Starch from maltose	40(28)	92	0	6
Starch hydrolysis	5	127	2	4
V.P. reaction	5	127	0	6
Haemolysis of sheep rbc	52	80	0	6

* Figures in the parentheses indicate number of strains showing late or weak positive reaction.

cases (30%) indicating that the majority of the organisms tested lacked this enzyme which decomposes the toxic peroxide. Eighty-one strains (59%) showed late positive reaction in the oxidase test while 20 strains (14%) gave a prompt positive reaction. Thirty-seven strains (27%) revealed no sign of the presence of cytochrome oxidase. Hippurate was hydrolysed to benzoate by 90 per cent. of 138 strains. With the exception of one in either case, all strains grew on MacConkey agar, but failed to grow in KCN broth. Hydrogen sulphide was produced by 34 strains (25%) only, the majority of strains failing to do so. One hundred and six strains (77%) reduced methylene blue mostly with a strong positive reaction, but with 25 showing a weak positive result. Generally starch forming properties of the organism were more marked on maltose agar than on glucose agar. Forty strains were able to produce iodophilic polysaccharide on maltose agar, of which 28 revealed weak positive reactions, while 7 out of 138 strains showed the ability to synthesize starch on glucose agar.

The results of the haemolytic activity of 138 strains of A. equuli examined are shown in Table 6 and individually given in Appendix A. Haemolysis on sheep blood agar plates incubated 24 to 48 hours was noted in 52 cases (38%) in the 138 strains investigated. On further incubation or storage at room temperature the

zone of haemolysis appeared to become more marked and in some cases it was similar in appearance to the haemolysis seen with Pasteurella haemolytica. The degree of haemolysis on blood agar appeared to be influenced by the species of red blood cells incorporated in the base medium. In general the haemolytic activity of the organism was more marked on sheep blood agar than on horse blood agar. Thirty (58%) of 52 strains which were haemolytic on sheep blood agar produced a zone of haemolysis on horse blood agar plate. On sheep blood agar twenty-five of 52 haemolytic strains developed a wide zone of complete haemolysis while others showed partial haemolysis which resembled in appearance that of Pasteurella haemolytica. Four of 30 strains which haemolysed horse red cells developed a zone of complete haemolysis on horse blood agar while the others produced a narrow zone of partial haemolysis.

The appearance of haemolytic capability, although infrequent, deserves mention. Two strains which were isolated from endotracheal swabs taken from apparently healthy horses were initially non-haemolytic both on sheep blood and on horse blood agar. A group of 30 strains including these two had been passaged at approximately three-weekly intervals in cooked meat medium for about 6 months and were then replated on sheep blood agar not only to test their purity and viability but also to get day-to-day working cultures. The two strains,

RDV24 and ERS55, then showed haemolysis. The representative colony was propagated in cooked meat medium and plated on layered horse and sheep blood agar plates. A partial zone of haemolysis was observed around colonies on both plates. There was, however, no change in the biochemical and fermentative properties of these two strains.

None of the strains appeared to produce any diffusible pigment in nutrient agar. However, it is worth noting that five haemolytic strains isolated from diseased pigs seemed to produce a certain pigment which was not easily differentiated from the colour of nutrient agar or milk agar. This colouration of the organism was clearly seen however when the bacterial growth on nutrient agar was harvested and washed with saline solution. The saline suspension of 5 haemolytic porcine strains (EQ9, 10, 11, 12 and 13) exhibited a creamy yellow colour while the others gave a suspension of greyish white colour seen with most non-pigment producing organisms.

Detailed examination of the results showed on the whole few differences between strains from healthy and diseased horses (Tables 7 and 8). A comparison of strains from horses and pigs is made in Tables 9 and 10 which show no differences in their biochemical and fermentative properties. All pig strains, however, failed to ferment sorbitol while 23 (18%) of 128 equine strains

Table 7

Fermentation reactions of A. equuli strains from healthy and diseased horses

Fermentable substrate	Strains from pathological material (23)*	Strains from healthy animal (100)
Arabinose	26 **	37
Galactose	96	99
Mannose	87	93
Cellobiose	17	33
Maltose	100	99
Melibiose	91	96
Trehalose	91	96
Starch	13	18
Glycerol	91	92
Mannitol	83	67
Sorbitol	9	19
Salicin	17	36

* Number of strains studied indicated in the parentheses.

** Figures indicate the percentage of strains positive.

Table 8

Biochemical reactions of A. equuli strains from healthy and diseased horses

Test for	Strains from pathological material (23)*	Strains from healthy animal (100)
Catalase	26 **	29
Oxidase	83	70
Aesculin hydrolysis	17	37
Hippurate hydrolysis	87	91
H_2S production	48	21
Gelatin(plate) hydrolysis	4	0
Growth in KCN broth	4	0
Growth on MacConkey agar	100	99
Methylene blue reduction	70	83
Starch from glucose	4	6
Starch from maltose	26	34
V.P. reaction	13	2
Haemolysis of sheep rbc	17	40

* Number of strains studied indicated in the parentheses.

** Figures indicate the percentage of strains positive.

Table 9

Fermentation reactions of 128 equine and 10 porcine strains of A. equuli

Fermentable substrate	Equine strains		Porcine strains	
	No.of strains positive	Percentage of strains positive	No.of strains positive	Percentage of strains positive
Arabinose	43	34	5	50
Xylose	128	100	9	90
Galactose	126	98	9	90
Mannose	118	92	9	90
Cellobiose	39	31	5	50
Maltose	127	99	10	100
Melibiose	122	95	10	100
Trehalose	121	95	10	100
Raffinose	124	97	10	100
Starch	22	17	3	30
Glycerol	118	92	8	80
Mannitol	89	70	5	50
Sorbitol	23	18	0	0
Salicin	42	33	5	50

Table 10

Biochemical reactions of 128 equine and 10 porcine strains of A. equuli

Test for	Equine strains		Porcine strains	
	No. of strains positive	Percentage of strains positive	No. of strains positive	Percentage of strains positive
Catalase	36	28	4	40
Oxidase	92	72	9	90
Aesculin hydrolysis	43	34	5	50
Hippurate hydrolysis	116	91	8	80
Gelatin(plate) hydrolysis	1	1	1	10
H_2S production	32	25	2	20
Growth in KCN broth	1	1	0	0
Growth on MacConkey agar	127	99	10	100
Methylene blue reduction	103	81	9	90
Nitrate reduction	128	100	9	90
Starch from glucose	7	6	0	0
Starch from maltose	40	31	0	0
Starch hydrolysis	7	6	0	0
V.P. reaction	5	4	0	0
Haemolysis of sheep rbc	47	37	5	50

broke down this substrate promptly.

In Table 6, there are no marked differences in most of the biochemical reactions of the 132 strains examined in the present study and those of the 6 strains obtained from the National Collection of Type Cultures. With the tests for aesculin hydrolysis and for the haemolysis of sheep red cells, however, minor differences in those properties were observed between the two groups. The six NCTC strains were negative in both tests. Among the remaining 132 strains from various workers, forty-eight (35%) had the ability to hydrolyse aesculin within 1 to 7 days and fifty-two strains (38%) revealed haemolytic activity on sheep blood agar. A close relationship between the abilities to hydrolyse aesculin and the haemolytic properties of the organism was noted in the majority of the strains showing these characters.

The comparative fermentative and biochemical properties of haemolytic and non-haemolytic strains of A. equuli are presented in Tables 11 and 12. Differences in the fermentative reactions between these two groups were noted with cellobiose, mannitol and salicin. No fermentation of either cellobiose or salicin was obtained with any of the non-haemolytic strains, but on the contrary the majority of haemolytic strains fermented cellobiose and salicin with rates of 85 and 90 per cent., respectively. Marked discrepancies between haemolytic and non-haemolytic strains in mannitol fermentation

Table 11

Comparative fermentation reactions of haemolytic and non-haemolytic A. equuli

Fermentable substrate	Non-haemolytic strains (86)*	Haemolytic strains (52)
Arabinose	33 **	39
Xylose	99	100
Galactose	97	100
Mannose	97	92
Cellobiose	0	85
Maltose	99	100
Trehalose	93	98
Raffinose	95	100
Starch	16	21
Glycerol	88	96
Mannitol	99	17
Sorbitol	19	14
Salicin	0	90

* Number of strains studied indicated in parentheses.

** Figures indicate the percentage of strains positive.

Table 12

Comparative biochemical and cultural properties of haemolytic and non-haemolytic A. equuli

Test for	Non-haemolytic strains (86)*	Haemolytic strains (52)
Catalase	33 **	23
Oxidase	76	69
Aesculin hydrolysis	1	90
Hippurate hydrolysis	91	89
H_2S production	19	35
Gelatin(plate) hydrolysis	2	0
Growth in KCN broth	1	0
Growth on MacConkey agar	99	100
Methylene blue reduction	83	67
Nitrate reduction	100	98
Starch from glucose	5	6
Starch from maltose	37	15
Starch hydrolysis	6	4
V.P. reaction	2	6

* Number of strains studied indicated in the parentheses.

** Figures indicate the percentage of strains positive.

were also noted. Although 99 per cent. of non-haemolytic strains fermented mannitol only 17 per cent. of haemolytic strains exhibited the ability to break down this alcohol. Aesculin was hydrolysed by no less than 90 per cent. of the haemolytic strains while only one of 86 non-haemolytic strains showed the ability to hydrolyse the glycoside by 7th day of observation. No differences in other biochemical and fermentative reactions were found between these two groups.

Correlation between some biochemical and fermentative characters, i.e. cellobiose, mannitol and salicin fermentation and aesculin hydrolysis, and haemolytic ability of A. equuli is presented in Table 13. Eighty-four (98%) of 86 non-haemolytic strains failed not only to ferment cellobiose or salicin but also to hydrolyse aesculin while fermenting mannitol mostly with prompt reaction. On the other hand all organisms which fermented salicin or cellobiose exhibited haemolytic activity and hydrolysed aesculin. The majority of haemolytic strains fermented cellobiose and salicin and hydrolysed aesculin, while failing to break down mannitol within 14 days of incubation.

The comparative biochemical and cultural characters of 5 porcine haemolytic strains and 47 equine haemolytic strains are presented in Table 14. The most striking difference between these two groups was the ability of porcine strains to develop creamy yellow colouration of

Table 13

Correlation between some biochemical properties and haemolytic capability of A. equuli

Fermentation of			Hydrolysis of aesculin	No. of strains showing	
Salicin	Mannitol	Cellobiose		Haemolysis	Non-haemolysis
-	-	-	-	3(5.8)	1(1.2)
-	+	-	+	1(1.9)	1(1.2)
-	+	-	-	1(1.9)	84(97.7)
+	+	-	-	1(1.9)	0
+	+	-	+	2(3.8)	0
+	+	+	+	4(7.7)	0
+	-	+	+	40(76.9)	0

Figures in the parentheses indicate the percentage of strains giving indicated reaction pattern.

+ = positive reaction - = negative reaction

Table 14

Comparative biochemical and fermentative characters of haemolytic A. equuli from horses and pigs

Test for	Equine strains (47) *	Porcine strains (5)
Catalase	17 **	80
Oxidase	68	80
Aesculin hydrolysis	89	100
Hippurate hydrolysis	91	60
H_2S production	36	20
Methylene blue reduction	74	0
Nitrate reduction	100	80
Starch hydrolysis	4	0
Starch from glucose	6	0
Starch from maltose	17	0
V.P. reaction	6	0
Pigment production	0	100
Fermentation of		
Arabinose	32	100
Mannose	91	100
Cellobiose	83	100
Melibiose	98	100
Trehalose	98	100
Starch	23	0
Glycerol	96	100
Mannitol	19	0
Sorbitol	15	0
Salicin	89	100

* Number of strains studied indicated in the parentheses.

** Figures indicate the percentage of strains positive.

their colonies on nutrient agar, while equine strains appeared not to produce any pigment. Porcine strains were more active in catalase reaction than the equine strains, their rate of positive reaction being 80 and 17 per cent., respectively. On the contrary, seventy-four per cent. of equine strains showed positive or weak positive reactions in the methylene blue reduction test while no porcine strain revealed a positive reaction in this test. In general, the fermentative reaction of porcine strains was more vigorous than equine strains and comparatively constant, but minor variations in fermentative activity were observed between equine haemolytic strains. Considerable differences between these two groups were noted in the fermentation of arabinose. Every porcine strain fermented arabinose within 24 hours but only 32 per cent. of equine strains produced acid from arabinose but with delayed reaction. Some minor differences between these two groups were also observed in the fermentation tests with mannitol, sorbitol, cellobiose and salicin. The biochemical and fermentative properties of the 5 porcine haemolytic strains resembled those of A. suis studied by Mair et al. (1974), although minor differences between them were observed in the tests for catalase production and nitrate reduction.

4. DISCUSSION

(a) Morphological characters

In general, the morphological findings of A.equuli examined in the present study agree with those described in the literature. It is generally accepted that the usual morphology of the organism consists of mostly short bacillary forms but characterised by the lack of uniformity in its form, especially in the length of the organism. Meyer (1910), who found the bacillus in smears made from the viscid pus of a kidney abscess and lung nodules, described it as "a rather long, thick bacillus with rounded ends", the length varying between 0.5 to 0.8 µm. Finding that, after repeated sub-cultivation, the form of the bacillus changed to that of pleomorphic rods and even filaments, he considered that the morphological variations of the bacillus were more pronounced in cultures than in tissues.

Most of the subsequent workers generally agreed that the pleomorphism is one of the characteristic features of the organism (Edwards, 1931; Hirato, 1939; Cottew and Ryley, 1952; Sakazaki and Watanabe, 1956; Hutchinson, 1956; Wetmore et al., 1963; Phillips, 1966; Mráz, Zakopal and Matousek, 1968). On the other hand, Magnusson (1919) recorded that the morphology of the organism was rather constant in its form. He remarked upon the uniformity of the bacillus either directly from

kidney foci or from fresh cultures. Snyder (1925) also observed uniformity in the form of the organism with her strains. Ashford and Shirlaw (1962) noticed mainly short bacillary forms on the films from the lesions of endocarditis in a piglet, and Carter, Marshall and Jolly (1971) also observed similar forms on direct smears from the liver, kidney and spleen of a foal. The organism obtained from Ashford and Shirlaw (1962), which was subcultured several times at least since its isolation from the lesion of verrucose endocarditis, was investigated in the present work, and found to be pleomorphic in its morphology. Moreover, variation in morphology of the organisms in the present series was noted with the medium on which the organism was grown. In view of these findings, the pleomorphism of the organism, considered by many workers to be a prominent morphological character of A.equuli, may not be a reliable criterion in the identification of the organism from pathological materials. The morphological variation of the organism appears to be more pronounced in the cultures than in the tissues, since primary cultures often failed to show pleomorphism, while this was seen with most laboratory strains.

The bipolarity of the organism observed by Sakazaki and Watanabe (1956) was noted with the organisms in the present investigation. Mostly the ends of coccobacillary forms were more deeply stained than the central part,

imitating the appearance of Pasteurella multocida and sometimes looking much like diplococci. But the bipolarity was never seen with the long or swollen rods often found in cultures grown on glucose or maltose agar.

The granules, regarded by Phillips (1961, 1966) to be characteristic of A. lignieresi and A. equuli, were observed in most cases of the present series. Magnusson (1919), describing the morphology of the organisms isolated from foal, mentioned that "In both old and young cultures, either in solid or liquid media, one can always discern the oval form". The granule of A. lignieresi and A. equuli originally described by Phillips (1961, 1966) may be the same structures as the 'oval form' of A. equuli observed by Magnusson (1919). Since no such structures have been observed in other Gram-negative bacteria, the constant presence of the granules in the cultures of the organisms investigated in the present study appears to be a valuable criterion in the primary identification of the organism.

Edwards (1931) described unusual forms met with in young agar cultures of the organism. Large yeast-like bodies, long or filamentous forms and streptococcus-like chains were observed with both smooth and rough strains. After 24 hours incubation the rough culture was composed entirely of short bacillary forms, while unusual forms were still numerous in the smooth culture.

So these unusual forms were considered by him to occur more commonly in the smooth than in the rough strains. Similar bizarre forms described by Edwards (1931) were noted with the strains in the present series, but there was no marked difference between smooth and rough strains as far as these unusual forms were concerned. No previous workers have described either the round bodies, simulating forms met with in the pleuropneumonia group, or swollen rods observed with the strains in the present study. It may be worth trying to find out whether L phase variation occurs spontaneously with this group of organisms, since unusual pleomorphic forms were observed in cultures of A. equuli employed in the present work. Dienes and Edsall (1937) noted the resemblance between Actinobacillus actinoides and Streptobacillusmoniliformis. The fact that Streptobacillus moniliformis is closely related to actinobacilli, led Wilson and Miles (1964, p. 518; 1975, p. 548) to propose to call it Actinobacillus muris (A. moniliformis).

Meyer (1910) observed a swollen irregular mucous capsule with his organism treated with dilute acetic acid, but capsules have not been demonstrated by the majority of subsequent workers. No evidence of capsules was observed in any of the cultures examined in the present study, although the extracellular slime said to be composed of mucus material (Cowan, 1974, p. 96)

was demonstrated either in wet India ink preparations or in the stained smears of ropy sediments formed in broth cultures.

(b) Cultural characters

One of the interesting characters of the organisms examined in the present study was the stickiness of their colonies grown on agar medium which made them difficult to remove from the agar surface and, on hooking them with an inoculating loop, cause them to pull out like elastic. The stickiness of colonies of A. equuli has been considered to be a characteristic feature of the organism by Meyer (1910), Magnusson (1919), McFadyean and Edwards (1919), Lütje (1921, 1922a), Snyder (1925), Edwards (1931), Hirato (1939), Cottew and Ryley (1952) and Phillips (1966).

Lütje (1921, 1922a) noted that the organism, when originally isolated, formed tenacious colonies but that after continued subculture the bacilli produced a more moist, mucoid type of colony. On further subculture the mucoid character of the growth was even lost entirely. Beller (1924) and Clarenberg (1925) also noted the loss of mucoid character of the organism on subculture.

Edwards (1931) considered that changes in the mucoid properties of the organism were closely related to the phenomenon of rough and smooth variation of colonial forms. Rough colonies were always mucoid while non-

mucoid colonies were invariably smooth. A. equuli in most cases was isolated as the rough, mucoid type. On continued subcultivation the roughness of the colonies gradually disappeared and eventually the bacillus developed smooth forms. With this change in colony form the mucoid property was gradually lost. On the contrary, Goerttler (1925), finding the mucoid character so constantly with his organisms, has denied the existence of a non-mucoid form of A. equuli.

Two colonial types, rough or smooth, have been observed with the organisms studied in the present work. Rough strains were always tough and tenacious and the majority of the smooth strains produced sticky colonies on agar medium, non-sticky growth was observed in a few cases. In view of these, sticky growth of the organism appears to be a useful criterion in the primary identification of the organism.

Snyder (1925), presenting the first report on the isolation of A. equuli in the United States of America, stated that "Its outstanding characteristic, that of slime production, is a considerable aid in distinguishing its colonies from those of any other bacterium". This slime material was demonstrated by Phillips (1966) in the Gram-stained smears in the form of a faintly staining interstitial substance. This extracellular slime was more clearly demonstrated in the present study in the films from ropy sediments developed in the broth as

faintly staining amorphous substance. The existence of extracellular slime, mainly composed of mucoid substances, appeared to be closely related not only to the sticky growth of the organism but also the viscous nature of its broth culture. Strain NCTC3365, the colonies of which were not sticky and failed to produce ropy sediment in broth culture, has hardly shown any evidence of the presence of slime, while strain NCTC9435, which appeared to be the stickiest in the present series, was one of the richest slime producers. The agar colonies of NCTC9435 were so firmly adherent to the medium that the removal of its growth from the agar plate was extremely difficult. The broth culture of this strain was also extremely ropy, the whole medium often being changed to a mucoid substance. The adherence of colonies to the medium and the ropiness of the broth culture appeared to be dependent upon the amounts of slime materials produced by the organisms during their multiplication. However, no attempts were made in the present work to quantify the slime material produced by the growing organisms.

(c) Low vitality of the organism

The low vitality of A. equuli in culture media has been observed in the present work, dying out mostly within 10 days or so either in nutrient broth or on blood

agar cultures kept at room temperature. This low vitality of the bacillus has also been observed by many previous workers and regarded as one of the distinguishing characters of the organism. Meyer (1910), emphasising the difficulty of maintaining the cultures, described that serial subcultures should be made to preserve the organism every 2 to 3 days, otherwise there was always the risk of the strain dying out. The survival time of the cultures on different media held at room temperature has been variously estimated at from 4 days to 4 months (Meyer, 1910; Magnusson, 1919; Edwards, 1931; Hirato, 1939; McCollum and Doll, 1951; Cottew and Ryley, 1952). Thus, stress has been laid on the necessity for frequent subculture to maintain the organism (Edwards, 1931). However, Snyder (1925) reported that the viability of the organism could be prolonged by growing them in gelatin stabs or chopped meat medium at room temperature, always keeping away from the light. Under such conditions she was able to keep the organisms alive for 3 to 4 months. McCollum and Doll (1951) described a semi-solid infusion agar for preserving the organism, in which the cultures had been maintained in a viable state for 3 months. Some cultures of the organisms investigated in the present work have been maintained alive in cooked meat medium for 6 weeks, while others have died out in the same medium at 24 days.

A. equuli appears to have little resistance to

physical and chemical agents. It has been found that the organisms were easily killed by desiccation, pus dried at 37°C for 24 hours having proved to be sterile (Magnusson, 1919). Heating at 52°C for 5 minutes killed the organism in a pus sample as well as in broth culture (Magnusson, 1919). Gourvitch (1931) reported that the organism was killed immediately by 1 per cent. creolin, 0.25 per cent. corrosive sublimate or 2 per cent. phenol and in 5 minutes by 0.5 per cent formalin. Snyder (1925) found that the organism was very sensitive to exposure to sunlight. Agar cultures exposed to the sun for 10 or 15 minutes proved to be sterile. Hirato (1939) found that 1 in 5000 malachite green was bacteriostatic for this organism. He also noted that the mucoid forms of the organism showed a higher resistance than the non-mucoid strains. The ability of the organism to produce slime may to a certain extent be utilised as a weapon in the struggle for existence.

(d) Biochemical characters

The results obtained in the biochemical tests agree in large part with those recorded by previous workers whose results are summarised in Table 15 (p. 107). All organisms examined fermented glucose without gas production. Although Dickinson and Mocquot (1961) recorded the isolation of gas producing actinobacilli from the intestinal contents of normal pigs, all other workers

obtained fermentation of carbohydrates without gas production. The fermentation of glucose, fructose, sucrose and lactose has been observed by all those who recorded the use of these substrates, although fructose was not fermented by one strain examined by Vallée et al. (1963). Similarly, positive results in the majority of cases were obtained with xylose, mannose, galactose, maltose, raffinose and trehalose by all the previous investigators with the exception of Cottew and Francis (1954) whose strains failed to ferment galactose and trehalose with the proportion of 40 and 30 per cent. of their strains, respectively. The results with fermentation of galactose obtained by Cottew and Francis (1954) whose organisms were isolated from normal horses, are somewhat surprising since all previous workers had agreed with the results obtained by Edwards (1931) when his 40 strains fermented this sugar. In the present study 98 per cent. of 138 strains fermented galactose and there appeared to be no discrepancies between strains from normal and diseased animals, and almost the same results were obtained by most workers (Ubertini, 1933; Haupt, 1934; Hirato, 1939; Vallée et al., 1963; Frederiksen, 1973; Mair et al., 1974).

Trehalose was fermented by 95 per cent. of strains in the present series, and similar results were recorded by Hirato (1931), Sakazaki and Watanabe (1956), Phillips

(1966), Frederiksen (1973) and Mair et al. (1974). Although the non-fermentation of maltose, seen with only one strain in the present study, has been recorded by Osbaldiston and Walker (1972), all other workers have reported the fermentation of this sugar. Similarly, positive results were obtained with xylose in fermentation tests by almost all workers, which is matched with the present findings, although Carter, Marshall and Jolly (1971) and Baker (1972) each reported negative results with their single strain.

The late fermentation of mannose and galactose, seen with 61.9 and 22.5 per cent., respectively, of the strains of the present series, was observed by Cottew and Ryley (1952), Frederiksen (1973) and Mair et al. (1974) whose strains fermented these sugars within 3 days. With glycerol, disagreement in the results by different workers are noted, Magnusson (1931), Meixner (1931), Ubertini (1933), Haupt (1934) and Vallée et al. (1963) having obtained negative results with their strains, Edwards (1931), Cottew and Ryley (1952), Mráz, Zakopal and Matoušek (1968) and Cutlip et al. (1972) late positive reactions, and Hirato (1939), Cottew and Francis (1954) and Mair et al. (1974) variable results with mostly late fermentation. In the present study, 91 per cent. of the strains examined fermented glycerol mostly with late reaction, and these findings are in accordance with the results

reported by Hirato (1939), Cottew and Francis (1954) and Phillips (1966).

Only a limited number of workers have examined the fermentative activity of A. equuli with melibiose or cellobiose. Wetmore et al. (1963), Cutlip et al., (1972), Frederiksen (1973) and Mair et al. (1974), who recorded the use of melibiose in the fermentation test, obtained positive reactions and these results are in agreement with the present findings where 96 per cent. of 138 strains in the work consistently fermented the sugar. Considerable discrepancies in the fermentative reaction with cellobiose were noted between strains used in the present study, but Frederiksen (1973) and Mair et al. (1974) reported negative results with this substrate, whilst Mráz, et al. (1968) obtained positive reaction with their 3 haemolytic strains isolated from mares. It is interesting to note that cellobiose positive strains in the present investigation showed haemolytic activity on sheep blood agar while non-haemolytic strains failed to ferment this sugar. Correlation between these two and other properties of the organisms studied will be discussed in the latter part of this section.

With arabinose, mannitol and salicin, differences in the results are noted by most of the previous workers. Positive results with arabinose were recorded by Meixner (1931), Ubertini (1933), Cottew and Francis (1954) and Cutlip et al. (1972), while others obtained

negative reactions with this sugar (Magnusson, 1919; Lesbouryies, 1945; Hutchinson, 1956; Ross *et al.*, 1972). It was considered by Mair *et al* (1974) that *A. equuli* could be distinguished from *A. suis* by its ability to ferment mannitol and its failure to cause haemolysis, to produce acid from arabinose, cellobiose and salicin or to hydrolyse aesculin.

In the present investigation 35 per cent. of the organisms employed fermented arabinose, a similar result also having been observed by Edwards (1931), Haupt (1934), Hirato, (1939), Sakazaki and Watanabe (1956) and Frederiksen (1973). No differences in the fermentative reaction with arabinose were found between haemolytic and non-haemolytic strains examined in the present work. However, considerable discrepancies in the fermentative reaction with arabinose were noted between haemolytic strains from horses and those from pigs. Every porcine haemolytic strain attacked arabinose within 24 hours but only 32 per cent. of 47 equine haemolytic strains did so but with delayed reaction.

From a review of the literature, it is apparent that the majority of mannitol positive strains examined by previous workers failed to ferment salicin while salicin positive strains appeared not to attack mannitol (Magnusson, 1919, 1931; Hirato, 1939; Svenkerud and Iverson, 1949; Cottew and Ryley, 1952; Hutchinson, 1956; Sakazaki and Watanabe, 1956; Phillips, 1966; Frederiksen,

1973; Ross et al., 1972; Mair et al., 1974), although both mannitol and salicin positive strains have been recorded by Edwards (1931), Ubertini (1933), Cottew and Francis (1954) and Mráz et al., (1968) with a few of the strains they examined. Furthermore, Hirato (1939) described two biochemical types of A. equuli which could be differentiated by mannitol fermentation. According to the present results, 93 per cent. of mannitol positive strains failed to ferment salicin while 91 per cent. of mannitol negative strains produced acid from salicin. On the contrary, 95.6 per cent. of salicin-negative strains had the ability to produce acid from mannitol while 85.1 per cent. of salicin-positive strains failed to attack mannitol. No previous record of this organism failing to ferment both mannitol and salicin (seen with 4 strains in the present work) was found, although Edwards (1931) and Hirato (1939) considered that strains may differ in their fermentative reactions with some other carbohydrates.

Although sorbitol was not fermented by the strains examined by the majority of previous workers, the fermentation of this substrate was recorded by Ubertini (1933), Cottew and Ryley (1952), Ashford and Shirlaw (1962), Wetmore et al. (1963), Phillips (1966) and Ross et al., (1972). In the present investigation some 18 per cent. of the strains showed their ability to produce

acid from this sugar-alcohol, which is in general agreement with the results recorded by Wetmore et al. (1963) and Phillips (1966).

The fermentation of dextrin by all the strains investigated in the present work showed a marked difference from the results reported by Magnusson (1919), Meixner (1931), Haupt (1934) and Lesbouyries (1945); all their strains failed to ferment this polysaccharide. Variable results with the substrate were recorded by Edwards (1931), Cottew and Francis (1954) and Mair et al. (1974), while the consistent positive reactions seen in the present work were obtained with the strains examined by Vallée et al. (1963), Phillips (1966) and Mráz et al. (1968). Moreover, Cowan and Steel (1965, p. 13) have recommended against the use of dextrin as a substrate in the fermentation test because of its variable composition. In the view of these workers the ability of A. equuli to ferment dextrin may not be a useful criterion in the identification of the organism.

The non-fermentation of dulcitol and inositol seen with all strains in the present study has been noted by all previous workers, with the exception of Meyer (1910), who recorded fermentation of dulcitol. Similarly, there is almost complete agreement on the non-fermentation of rhamnose, inulin and adonitol, which is consistent with the present findings, although minor variations were recorded with these substrates by Edwards (1931)

Vallée et al (1963) and Phillips (1966).

Although some minor differences were observed in the fermentative characters of the organisms examined, the biochemical properties were comparatively constant. The lack of catalase activity in A. equuli was considered by Mair et al. (1974) to be a characteristic feature of the organism and has been reported by different workers (Carter et al., 1971; Cutlip et al., 1972; Ross et al., 1972; Windsor, 1973). On the other hand, Wilson and Miles (1964, p. 521) and Cowan (1974, p. 94) have recorded a positive catalase reaction as typical of A. equuli. These however are not in agreement with the present findings where some 29 per cent. of the strains were catalase positive. Variable results have also been reported by Phillips (1966) and Frederiksen (1973). Four workers have reported on the oxidase activity of the organism, Carter et al. (1971) and Windsor (1973) recording negative results,while Steel (1961) and Osbaldiston and Walker (1972) obtained positive reactions. In view of these results, it is not surprising that the present findings should have recorded 73.2 per cent. of strains showing oxidase positive reaction.

The ability of the organisms to produce ammonia has been tested by very few workers, Edwards (1931), Haupt (1939) and Phillips (1966) recorded positive results with this test, but Mráz et al.

(1968) obtained negative results. Although a positive nitrate reaction has been obtained by all previous workers occasional strains may give a negative result. This was seen in the present work in which one single strain failed to reduce nitrate to nitrite. None of the strains examined in the work hydrolysed gelatin in stab cultures within a two-week period of incubation and similar findings have been reported by the majority of the previous workers (Magnusson, 1931; Edwards, 1931; Haupt, 1934; Neter, 1942; Wetmore et al., 1963; Mráz, et al., 1968; Ross et al., 1972; Mair et al., 1974), although Meyer (1910), Frederiksen (1973) and Vallée et al. (1974) recorded positive results in the gelatin test with some of their strains. However, with Frazier's (1926) test, two strains in the present series showed gelatinase activity on the gelatin agar plate, and this method is considered by Phillips (Personal communication) to be more sensitive than the gelatin stab test.

Hydrolysis of aesculin by A. equuli has not been recorded by previous workers, but some 35 per cent. of the strains investigated in the present work had the ability to hydrolyse this glycoside. It is of interest to note that the ability of the organisms to hydrolyse aesculin appears to be closely related to other biochemical properties such as the ability to produce acid from cellobiose and salicin and the haemolytic

capability of the bacilli. The activity of the organism to hydrolyse sodium hippurate has not been examined by any previous workers with the exception of Phillips (1974), who regarded it as a differential character between A. equuli and A. lignieresi. The majority of the strains examined reacted positively in this test. A positive urease result has consistently been recorded by all previous workers with the exception of Moon, Barnes and Higbee (1969), who observed negative reactions in the urease test with their two strains isolated from monkeys.

The failure of A. equuli to produce hydrogen sulphide was considered by Vallée et al. (1963) to be a differential feature between this organism and A. lignieresi and has been noted by many workers (Magnusson, 1931; Sakazaki and Watanabe, 1956; Phillips, 1966; Frederiksen, 1973; Mair et al., 1974). However, positive results with this test have also been recorded by Ubertini (1933), Haupt (1934), Hirato (1939), Cottew and Ryley (1952), Mráz et al. (1968) and Ross et al. (1972). There is universal agreement on the failure of the organism to produce indole. Similarly, negative results with the methyl red and Vöges-Proskauer tests have been noted by all previous workers with the exception of Edwards (1931), Svenkerud and Iverson (1949) and

Osbaldiston and Walker (1972), who recorded positive M.R. tests with their 1, 2 and 3 strain(s), respectively. The present findings of positive Vöges-Proskauer test with 5 strains in the work appear to be the first record. 96.4 per cent. of the strains examined, however, reacted negatively in this test.

The growth on MacConkey's medium was reported by Wetmore et al. (1963), Jones and Simmons (1971) and Mair et al. (1974), but Ashford and Shirlaw (1962) and Cutlip et al. (1972) each reported no growth of their single strain on this medium. Growth in KCN broth and citrate medium has not been recorded by any previous workers with the exception of Windsor (1973), who obtained citrate positive results with his pig strains, and Osbaldiston and Walker (1972), who isolated potassium cyanide-resistant strains from calves. The negative results obtained in the present work with malonate fermentation, phenylalanine deamination and gluconate oxidation support the findings of Frederiksen (1973), Windsor (1973) and Mair et al. (1974). β-galactosidase activity of the organism was examined by Frederiksen (1973) and Mair et al. (1974), and they observed positive results with all the organisms they examined.

The production of starch from glucose and maltose by A.lignieresi was considered by Phillips (1966) to be a characteristic feature of the organism, and he suggested that actinobacilli may form part of the iodophilic flora

1973) and Mair *et al*. (1974) to be a distinguishing feature between this organism and *A. suis*, some 37.7 per cent. of the strains examined in the present work were haemolytic on layered plates of sheep blood agar. It is well known that haemolysis can be seen better on layered blood agar plates (Cowan and Steel, 1965, p. 102), but no previous workers have examined the haemolytic activity of this organism in this way. The present findings with 52 haemolytic strains are of interest since all other workers have used either a limited number of tests or only small numbers of strains, mostly with 1 to 3 strains. The haemolytic activity of the organisms studied in the present work appeared to be more marked on sheep blood agar than on horse blood agar.

Carter *et al*. (1971) and Larsen (1974) reported the isolation of the haemolytic variants of *A. equuli* from foals with septicaemia or arthritis and from horses with chronic alveolar emphysema, and considered that the failure of their organisms to ferment mannitol was characteristic since non-haemolytic strains frequently fermented mannitol. Bell (1973) isolated an haemolytic *A. equuli* from a pig, and found the non-fermentation of mannitol was one feature of his organism. On the other hand, Mráz *et al*. (1968) recorded the isolation of 3 strains of haemolytic *A. equuli* which fermented mannitol, salicin and cellobiose.

Among 52 haemolytic strains examined in the present work, four strains (8%) were similar to those isolated by Mráz et al. (1968) while 43 (83%) strains resembled the haemolytic A. equuli described by Carter et al. (1971), Bell (1973) and Larsen (1974).

Cottew and Francis (1954) reported the isolation from mouth and cervical swabs from 59 horses of 30 strains of A. equuli, 12 of which were haemolytic on sheep blood agar while others showed no haemolysis on sheep blood agar on primary cultivation. Twenty-eight of 29 salicin-positive strains failed to produce acid from mannitol. Seventeen salicin-positive strains were non-haemolytic on primary isolation, although five of them showed haemolytic activity on continued subcultivation. However, all salicin-positive strains in the present series were haemolytic on layered sheep blood agar.

Wetmore et al. (1963) and Hughes and Murphy (1972) were of the opinion that the haemolytic ability of actinobacilli was a variable characteristic while others considered that this characteristic was permanent and unvarying (Zimmermann, 1964; Frederiksen, 1973; Mair et al. 1974). Although it is doubtful whether the ability of A. equuli to produce haemolysis should be weighted more than the ability to ferment any one carbohydrate or to hydrolyse aesculin, the haemolytic ability of the bacilli appeared to be one of the

outstanding features of the organisms investigated in the present study since some biochemical characters such as fermentation of mannitol, salicin and cellobiose, and hydrolysis of aesculin are closely related to this ability.

In the course of the present study on the fermentative and biochemical characteristics of A. equuli from various sources it has proved possible to place each strain examined so far into one of two main groups. One group, which was haemolytic on layered sheep blood agar, fermented cellobiose and salicin and hydrolysed aesculin in 85, 90 and 90 per cent. of cases respectively, while the other group which was non-haemolytic on layered sheep blood agar failed to ferment cellobiose and salicin and to hydrolyse aesculin. Considerable differences between these two groups were also observed with the fermentative ability against mannitol. Haemolytic strains mostly failed to produce acid from mannitol while non-haemolytic strains were mannitol fermenters.

The uniformity of the carbohydrate fermentation and biochemical properties could not be demonstrated in haemolytic strains isolated from horses although relatively constant results were obtained in haemolytic strains from pigs. The ability of porcine haemolytic strains to produce creamy yellow pigment is one of the most characteristic features of the organisms, although

the production of pigment by actinobacilli has not been recorded previously. Too much weight should not be attached to this result, however, since relatively few strains from pigs were available.

Part II: Studies on the antigenic structure and serological typing of *Actinobacillus equuli*

1. INTRODUCTION

It is apparent from a review of literature that only slight attention has been given to the serological characteristics of Actinobacillus equuli. Meyer (1910), presenting the very first record of A. equuli infection in a horse, described the use of a serum agglutination test, precipitin test and complement-fixation test for the diagnosis of the disease. He concluded that the serological diagnosis of the disease was difficult because of the inagglutinability due to the mucoid consistency of the organism and the non-specific reactions encountered with precipitin and complement-fixation tests.

However, serological investigations on A. equuli by subsequent workers have been carried out primarily to provide a basis for serological diagnosis and for serum therapy. Lütje (1922a) remarked that considerable differences existed in the agglutination of different strains with a polyvalent antiserum prepared for therapeutic purposes, and claimed that the strains were not serologically identical. Laudien (1923) and Kowatsch (1925) also noted the serological heterogeneity of their strains. Goerttler (1925) investigated the agglutinative properties of a number of strains, and noted that saline suspensions of agar cultures were unsuitable for use as antigens, owing to the viscous nature and inagglutinability of the organisms. By

treating the bacterial growth with 66 per cent. alcohol for 2 to 3 hours and suspending them in carbol saline, the organisms were rendered more agglutinable. He also noticed that those easily agglutinable strains readily produced agglutinins, while inagglutinable strains caused little or no rise in the agglutinative titres of the serum of the experimental animals. Noting a close correlation between the agglutinability and the agglutinogenic characteristics of his organisms, he emphasized quantitative differences in the agglutinability and the agglutinogenic power of the organisms. Thus, he found no qualitative antigenic differences, and concluded that the organisms were all closely related serologically. Likewise, Meixner (1931) considered that the species was serologically uniform.

Edwards (1931, 1932) studied more extensively the serological characteristics of 40 strains by means of the agglutination, precipitin, complement-fixation and agglutinin-absorption tests. Only three cases of serological identity were observed with the antisera prepared from seven strains. On the basis of these results he concluded that the strains investigated formed an extremely heterogeneous serological group. Hirato (1939) also found an extreme heterogeneity in the serological characters of his strains.

Miessner and Berge (1922), Langhoff (1923) and

Maguire (1958) have attempted to diagnose infection with A. equuli and to determine whether infection in foals was of prenatal origin by using the serum of the dam in the agglutination test, and they have claimed that infection could be diagnosed in this way. However, Edwards (1931) was of the opinion that it would be impossible to determine the presence of agglutinin in the serum unless the infecting organism had been isolated and was used as antigen in the tests.

Antigenic relationships between A. equuli and A. lignieresi have been observed by Haupt (1934), Vallée et al. (1963), Bouley (1966), Phillips (1966) and Mráz (1968). Furthermore, Wilson and Miles (1975) considered that A. equuli shares major antigens with A. lignieresi. Bouley (1966) observed an antigenic relationship between A. equuli and A. suis. Mráz (1968), finding marked cross-agglutination and precipitin reactions between A. suis and haemolytic variants of A. equuli, considered that A. suis studied by him represented only haemolytic variants of A. equuli. Haupt (1934), using the complement-fixation technique, observed a serological relationship between A. equuli and pseudomonas (Pseudomonas pseudomallei and Ps. mallei). On the contrary, Wetmore et al. (1963) found that Actinobacillus strains were remarkably different from either Ps. mallei or Ps. pseudomallei.

Miessner and Berge (1922) studied the serum of

30 dams whose foals had died of A. equuli infection. They found that, in five of 30 cases, an agglutination titre of 1 in 200 was obtained and in the remainder below 1 in 200 with a titre of 1 in 2,000 in the specific antiserum. Dimock and Edwards (1932) have reported the serum of normal mares usually agglutinates A. equuli at a dilution of 1 in 100, in many cases causes agglutination at 1 in 200, and rarely in even higher dilutions. In an attempt to demonstrate specific agglutinins in the blood of mares, Dimock et al. (1947a) tested the serum of 10 dams of infected foals, using the homologous organism as antigen. Finding that in none of these cases were there specific agglutinins for the infecting strains, they concluded that the serological diagnosis of the disease would hardly be practical.

Specific treatment of foals with an antiserum prepared from A. equuli or with dam's blood has been attempted but without encouraging results (Magnusson, 1919; Merchant and Packer, 1967). Maguire (1958) claimed that satisfactory protection of foals was obtained by vaccinating pregnant mares, but Doll (1963) and Knight (1972) considered immunoprophylaxis to be impractical because of the extreme antigenic diversity of A. equuli.

It is the purpose in this section of the work to determine the antigenic characteristics of the 138 strains of A. equuli described in Part 1 of the present

work and, possibly, to relate them to other properties of the organism.

2. MATERIALS AND METHODS

(a) Sources of strains

A total of 138 strains of *A. equuli* employed in this section of work were those whose morphological, cultural and biochemical characteristics have already been described in Part 1 of the present study, and the details of the strains used are presented in Appendix A.

(b) Media and reagents

Except where otherwise indicated the reagents and media used in the preparation of antigens were prepared as described by Cruickshank (1972).

(c) Preparation of antigens

(i) Bacterial suspensions for rabbit immunisation

a) Heated vaccine

Bacterial suspensions for use as immunising agents in the preparation of antisera were prepared by growing the organisms on the surface of nutrient agar slope in 120 ml. medical flat bottles inoculated with 2 ml. of an overnight broth culture. After 20 to 24 hours at 37°C the bacterial growth was washed off from the agar slope with 5 ml. of saline (0.85 per cent. sodium chloride solution) and filtered through the cottom wool fitted down into the shoulder part of Pasteur pipette. The cells were separated by centrifugation and resuspended in saline to make the original volume. This washed bacterial suspension was steamed at 100°C for 2 hours,

and then centrifuged at 2,200 x G for 25 minutes. The packed cells were resuspended evenly in sterile saline solution to a density corresponding to Brown's opacity tube no. 8 (Burroughs Wellcome).

b) Formolised vaccine

Nutrient broth (digest broth) cultures (20 to 24 hours) were centrifuged at 2,200 x G for 25 minutes and the packed cells were resuspended in 0.2 per cent. formolised physiological saline. The density was adjusted to Brown's tube no. 8 as for the heated vaccine.

(ii) Bacterial suspensions for agglutination tests

a) Slide agglutination test antigen

Bacterial suspensions for use as antigens in the slide agglutination test were prepared in the same way as the heated vaccine, except that cells were suspended in phosphate buffer (0.01M, pH 7.2) containing 0.85 per cent.sodium chloride (PBS). After steaming at 100°C for 2 hours cells were centrifuged and resuspended in PBS to give approximately a 10 per cent. bacterial suspension, and then agitated for 15 minutes by the use of electric shaker to make an even suspension. This thick bacterial suspension was used in slide agglutination tests and referred to as 'slide antigen'. To preserve the antigens one part of 0.1 per cent. merthiolate solution was added to 9 parts of bacterial suspension and stored at 4°C until required. In this

condition antigens were quite satisfactory at least for one year.

b) Tube agglutination test antigen

Bacterial suspensions for use as antigens in the tube agglutination tests were prepared in the same way as the heated slide antigens, except that the bacterial cells were suspended in 0.3 per cent. sodium chloride solution (see Kwapinski, 1972, p. 298) and that the final density was adjusted to a scale reading of 2.0 on an EEL portable colorimeter (Evans Electroselenium Ltd., England) using a green (OGRI) filter. The antigen was agitated in the bottle with glass beads by the use of electric shaker for 15 to 30 minutes immediately before use and preserved by the addition of merthiolate to a final concentration of 0.01 per cent.

(iii) Soluble antigens for immunodiffusion precipitin and passive haemagglutination tests

a) Heat-stable soluble antigens

A nutrient agar slope in a 120 ml. medical flat bottle was inoculated with 2 ml. of an overnight nutrient broth culture and incubated for 20 to 24 hours. The bacterial growth was washed off the slope with approximately 5 ml. of PBS, and filtered through cotton wool to get rid of agar debris. The agar-free bacterial suspension was centrifuged and the packed cells were washed once with PBS by centrifugation. The washed cells were resuspended in PBS to give a 10 per cent. bacterial

suspension and then steamed for 2 hours at 100°C. The clear supernatant obtained by centrifugation of this heat-treated suspension at 2,200 x G for 40 minutes was retained as 'heat extract' and used either in immuno-diffusion or passive haemagglutination tests. The heat extract was preserved by the addition of merthiolate to a final concentration of 0.01 per cent. and stored in a refrigerator at 4°C until use.

b) Westphal-type lipopolysaccharide (endotoxic somatic antigen)

The procedure employed was Carter and Rappay's (1963) modification and simplification of the phenol-water extraction method of Westphal, Lüderitz and Bister (1952). Bacterial suspensions were prepared in the same way as the suspensions for the heat extract, except that the cells were washed and resuspended in deionized water. Prior to treatment, the cell suspension was formolised at 4°C overnight by the addition of formaldehyde to a final concentration of 0.2 per cent. This formolised suspension was added with shaking to an equal volume of 90 per cent. phenol solution (w/v) which had been previously heated in a water bath at 68°C and the mixture maintained at this temperature for 30 minutes. Immediately after removal from the water bath the mixture was cooled to 4°C in a cold room and then centrifuged for 40 minutes at 2,200 x G. The water phase was collected and an equal volume of deionized water was added to repeat the extraction. After

thorough mixing in the water bath, the mixture was again incubated for 30 minutes at 68°C. The mixture was cooled and centrifuged again in like manner, and then the water phase was siphoned out. The two water phases were combined and dialysed in a visking tubing (Scientific Instrument Centre Ltd., London) first against running tap water overnight and then against deionised water for another 24 hours to remove phenol and small amounts of low molecular weight bacterial substances. The dialysed, slightly opalescent solution was centrifuged to discard the insoluble material. One ml. of a 30 per cent. sodium acetate solution was added to each 10 ml. of the dialysed solution prior to the addition of 6 volumes of cold absolute alcohol. After thorough mixing, the mixture was kept in a refrigerator overnight and then centrifuged. The supernatant was discarded and the precipitate washed first with cold absolute alcohol and then with equal parts of absolute alcohol and acetone. After washing with alcohol acetone, the precipitate was washed with ether, the residual ether being evaporated by placing the MacCartney bottle in a fume chamber for about 2 hours. The dried lipopolysaccharide was re-suspended in PBS to make the original volume of the bacterial suspension. The lipopolysaccharide in PBS was preserved by the addition of merthiolate to a final concentration of 0.01 per cent. and stored at 4°C until required. This lipopolysaccharide antigen was used

both in the immunodiffusion and in the passive haemagglutination tests. Although the techniques described by Westphal and Jann (1965) for removing bacterial nucleic acid and traces of protein from the lipopolysaccharides were not employed, the product was found to be satisfactory for the tests.

c) Ultrasonic extract

A thick bacterial suspension was prepared as for the heat-stable soluble antigen and treated in an M.S.E. 60 watt Ultrasonic Disintegrator (Measuring & Scientific Equipment Ltd., England). A four ml. volume of bacterial suspension was treated in a 20 ml. heat resisting glass tube (M.S.E.) using the 3/8 inch diameter transducer probe. In order to avoid a temperature rise in the suspension during the operation the tube was immersed in a beaker filled with crushed ice. After disintegration of cells for 15 minutes the ultrasonicated suspension was placed in a cold room at $4^{\circ}C$ overnight and then the cell debris was removed by centrifugation at 2,200 x G for 40 minutes. The translucent supernatant so obtained was preserved with 0.01 per cent. merthiolate and is referred to as 'ultrasonic extract'.

d) Saline extract

A bacterial suspension was prepared as for the heat extract, except that cells were resuspended in 2.5 per cent. saline solution. This hypertonic bacterial suspension was agitated by the use of an electric shaker

for an hour at room temperature, and then centrifuged at 2,200 x G for 40 minutes. The clear supernatant obtained is referred to as 'saline extract' and was preserved by adding merthiolate to a final concentration of 0.01 per cent.

e) Sodium desoxycholate extract

The procedure employed with minor modifications was the sodium desoxycholate extraction method of Barta (1963). A thick bacterial suspension prepared in the same way as the suspension for heat extract was extracted for 18 hours at 4°C by the addition of 20 per cent. sodium desoxycholate to give a final concentration of 0.5 per cent. The treated suspension was centrifuged at 2,200 x G for 40 minutes. The supernatant obtained was dialysed against PBS for 2 days at 4°C. The volume of dialysate was adjusted to the original volume of the bacterial suspension used and referred to as 'sodium desoxycholate extract'.

f) Alcohol extract

A thick bacterial suspension (10 per cent.) in 0.2 per cent. formolised saline was mixed with an equal volume of absolute alcohol and then the mixture was incubated at 37°C for 18 hours according to the method described by Namioka and Murata (1961). After centrifugation, the supernatant was mixed with 1.5 volumes of acetone and placed in a cold room at 4°C overnight. The precipitate formed was collected by centrifugation

and resuspended in PBS to make the original volume of the bacterial suspension.

(d) Preparation of antisera

Twenty-nine antisera were prepared in rabbits against A. equuli strains RDV1, RDV9, RDV28, RDV44, RDV45, RDV55, RDV62, RDV64, ERS3a, ERS12, ERS18, ERS20, ERS22, ERS29, ERS31, ERS37, ERS40, ERS46, ERS47b, ERS53, ERS54, EQ2, EQ4, EQ7, EQ^3, NCTC3365, NCTC8529, NCTC8644 and NCTC8987 using the heated vaccines. Six additional antisera were prepared against A. equuli strains RDV55, ERS12, ERS29, NCTC3365, NCTC8529 and NCTC 8644 using the formalised vaccines. One rabbit was employed for each vaccine.

Prior to inoculation, rabbits were bled to test for the natural antibodies against the antigens to be used for immunisation. Injections were made into the marginal vein of the right ear, the left ear being reserved for bleeding by venepuncture. The two different types of antisera were prepared with a series of eight intravenous injections of the corresponding antigen suspension twice weekly for four weeks at intervals of three to four days, the first and second doses being 0.25 and 0.5 ml., respectively, and thereafter 1 ml. Test bleedings were made 7 to 10 days after the last injection and, if the antiserum titre was satisfactory, further bleedings were made from the ear vein or by heart puncture. If

the titre proved to be unsatisfactory, however, further injections were given until suitable titres were obtained.

Blood was collected in sterile McCartney bottles and then allowed to clot at room temperature. The clot was loosened by shaking and the serum collected after overnight contraction at room temperature using a sterile Pasteur pipette. It was then centrifuged at 2,200 x G for 25 minutes to remove the cells. Antisera were stored in a deep-freeze cabinet at -20°C until required. Antisera for day-to-day working were preserved by the addition of merthiolate to a concentration of 0.01 per cent. and kept in a cold room at 4°C.

(e) Agglutination tests

(i) Slide agglutination test

Slide agglutination tests were carried out on a glass plate over a black background with oblique lighting. One drop of slide antigen was mixed with one drop of antiserum on a glass plate and observed for clumps after one minute at room temperature. Antisera used for the slide agglutination tests were diluted with PBS containing 0.01 per cent. merthiolate until they gave readily observable agglutination of the homologous slide antigen within one minute on a glass plate. In practice, a one in five dilution of each antiserum proved to be satisfactory for this purpose.

(ii) Tube agglutination test

Antiserum was diluted in 6 by 50 mm. test tubes using a two-fold dilution series from 1 in 10. One-half ml. of antigen suspension was added to 0.5 ml of diluted antiserum and incubated 18 to 20 hours in a water bath at 56°C. The titre of the antiserum was expressed as the reciprocal of the highest final dilution of antiserum at which clumping could be readily detected. An automatic syringe (see Herbert, 1970, p. 121) was used in the preparation of the antiserum dilutions and addition of the antigen suspension.

(iii) Absorption of antisera

Absorbing strains of A. equuli were grown overnight on nutrient agar slopes in the same way as for the preparation of antigens. The bacterial growth was washed off the slope with physiological saline and filtered through the cotton wool fitted down into the shoulder part of a Pasteur pipette. The dense bacterial suspension was centrifuged and then bacterial cells were washed twice with physiological saline. The washed bacterial cells were suspended in physiological saline and heated for 2 hours at 100°C. The heated bacterial suspension was centrifuged and the packed cells were resuspended in saline to give a 10 per cent. suspension. This bacterial suspension was used for the absorption of antisera.

One volume of bacterial suspension was thoroughly mixed with an equal volume of a 1 in 5 dilution of

antiserum to be absorbed. After incubation at 37°C for 2 hours with frequent shaking the antiserum-antigen mixture was allowed to stand overnight in the cold room at 4°C. and the absorbing cells were then removed by centrifugation.

A single-tube agglutination test with equal volumes of the absorbed antiserum and the tube antigen of the absorbing strain was used to show that absorption was complete. If the absorption was incomplete, the procedure was repeated. Although a single absorption of an antiserum often was effective to exhaust the antibodies, the double or two-step absorption procedure recommended by Edwards and Ewing (1972) was carried out routinely in the later part of the work

(f) Passive haemagglutination test

(i) Preparation of erythrocytes

Sheep blood was collected aseptically in an equal volume of modified Alsever's solution (Alsever and Ainslie, 1941; Muschel and Lowe, 1955), which had been sterilised by autoclaving at 5 pounds for 20 minutes. The blood was kept at 4°C and was used for up to 3 weeks. At the beginning of each day an aliquot was taken and the cells were washed three times with 6 volumes of physiological saline solution and then suspended in PBS to give a 10 per cent. erythrocyte suspension.

(ii) Treatment of erythrocytes with antigen

One volume of antigen was added to an equal

volume of 10 per cent. erythrocyte suspension and the cells suspended using a Rotamixer (Hook & Tucker Ltd., England). The mixture was incubated in a water bath at 37°C for an hour with frequent agitation and then centrifuged at 700 x G for 10 minutes. The supernatant fluid was discarded and packed antigen-treated erythrocytes were washed three times with PBS and finally resuspended in PBS to give a 0.5 per cent. antigen-treated-erythrocyte suspension. This red cell suspension was used within 2 days or until haemolysis was clearly evident.

(iii) Absorption of antiserum with untreated fresh sheep erythrocytes

In order to remove heterophile and anti-species antibodies, absorption of antiserum with untreated sheep erythrocytes was carried out by the method described by Davies, Crumpton, MacPherson and Hutchison (1958). The antiserum was inactivated at 56°C for 30 minutes, then cooled to room temperature. One volume of a 1 in 5 dilution of inactivated antiserum was added to an equal volume of 10 per cent. washed fresh cells in PBS, and this antiserum-erythrocyte mixture was shaken well using the Rotamixer. The antiserum-red cell mixture was incubated in a waterbath at 37°C for one hour, during which time it was frequently shaken, after which it was centrifuged to sediment the cells. The

supernatant was used in the passive haemagglutination test as representing a 1 in 10 dilution of erythrocyte-absorbed antiserum. An occasional batch of sheep cells was found to be agglutinable to low titre (1 in 20) by some rabbit sera. This did not interfere with the interpretation of the tests since adequate controls were included and the antiserum dilutions used were beyond the range of the activity of these normal agglutinins. In view of these findings, antiserum without absorption with sheep cells was used in most cases.

(iv) Performance of passive haemagglutination test

Two-fold serial dilutions from 1 in 20 of the antiserum to be tested were made in PBS in a perspex W.H.O. haemagglutination plate in 0.4 ml volumes. An equal volume of 0.5 per cent. suspension of antigen-treated erythrocytes was added to each well. Two control wells were used: one containing 0.4 ml of PBS and 0.4 ml of 0.5 per cent. suspension of antigen-treated erythrocytes and the other containing 0.4 ml. of 1 in 40 dilution of antiserum and an equal volume of 0.5 per cent. suspension of washed fresh erythrocytes. The haemagglutination plate was shaken to get even mixing and then left at room temperature for 2 hours, at which time a preliminary observation was taken. The plate was then left overnight in the cold room at $4^{o}C$ and the final reading was made the following morning. On some occasions

further incubation at room temperature for 2 to 3 hours was found to allow satisfactory reading of the degree of agglutination.

The haemagglutination pattern rating used throughout the experiments was as follows:

4, homogeneous mat of cells on the entire bottom of well.

3, slight clear area around the edge of mat.

2, band of cells near the edge of mat.

1, agglutination around the central button or ring.

0, no agglutination, discrete button or ring of cells in the centre of well.

(), 'falling in' of the edges of mat in wells with haemagglutination.

The titre of haemagglutination was estimated in terms of the highest final dilution of antiserum showing homogeneous mat of antigen-coated erythrocytes on the entire bottom of well (haemagglutination pattern rating 4), whereas no haemagglutination should be observed in all control wells.

The amount or dilution of antiserum in the end-point well denoted one haemagglutination unit (dose).

(v) Haemagglutination-inhibition test

Serial two-fold dilutions of the antigen to be tested were made in volumes of 0.2 ml. in PBS in an haemagglutination plate; to each was added an equal volume of antiserum containing 4 haemagglutinating units of

Table 17

Diagram of the haemagglutination-inhibition test

Reactant	1st row	Control		
		1	2	3
Antigen dilution, from 1 in 10 to 1 in 10240, ml.	0.2	-	0.2	-
Phosphate buffered saline (0.01M, pH 7.2), ml.	-	0.2	0.2	0.4
Antiserum (4 HA units) ml.	0.2	0.2	-	-
Incubation at room temperature for an hour				
0.5% suspension of modified cells, ml.	0.4	0.4	0.4	0.4
Haemagglutination after 2 hour at room temperature & overnight at 4°C.*	0-4	4	0	0

* The figures indicate the degree of haemagglutination.

antibody. After incubation at room temperature for an hour, 0.4 ml. of 0.5 per cent. antigen-treated-erythrocyte suspension was added to each well and the whole test completed in the same way as a normal titration of haemagglutinating antibody. The titre of the haemagglutination-inhibition test was expressed as the final dilution of the antigen in the last well showing no agglutination of antigen-treated erythrocytes.

Three controls containing 0.2 ml. of antiserum (4 haemagglutinating units), 0.2 ml. of PBS and 0.4 ml. of 0.5 per cent. antigen-treated-erythrocyte suspension; 0.2 ml. of 1 in 5 dilution of antigen, 0.2 ml. of PBS and 0.4 ml. of 0.5 per cent. fresh red cell suspension; 0.4 ml. of PBS and an equal volume of 0.5 per cent. antigen-treated-erythrocyte suspension, respectively, were included in the tests. The schedule of the haemagglutination-inhibition test is diagramatically illustrated in Table 17.

(g) <u>Immunodiffusion precipitin test</u>

Immunodiffusion precipitin tests employed in the work were those described by Ouchterlony and Nilsson (1973). The agar gel consisted of 4.0 gm. sodium chloride, 0.7 gm. disodium phosphate, 0.6 ml. of N/1 hydrochloric acid and 5 gm. Difco Noble agar in 500 ml. of deionized water. After dissolving the agar, merthiolate was added to make up 0.01 per cent. A

volume of 4 ml. or 15 ml. of melted agar gel was pipetted on to a clean microscopic slide (76 x 25 mm.) or thin photographic plate (80 x 80 mm.), respectively, and allowed to harden at room temperature for about 15 minutes. Five or seven wells, 4 mm. in diameter and 3 mm. from the nearest edge of the centre well to those of outer wells, were cut by using the gel cutter (Feltham, England), and the agar was carefully aspirated from the wells with a Pasteur pipette attached to a Venturi water pump. The wells were then filled using a capillary Pasteur pipette, antisera were placed in the outer wells, and antigen in the centre well or vice versa. The slides or plates were placed in a large square Petri dish lined with a piece of moist filter paper and supported on two glass rods to prevent contamination by water. The results were examined after 24 hours at room temperature. For a permanent record the precipitation slide was fixed and stained by the method described by Scheidegger (1955).

(h) Immunoelectrophoresis

Immunoelectrophoretic analysis of antigens of A. equuli was carried out by the method described by Grabar and Burtin (1964). The test antigens were electrophoresed in a Shandon Electrophoresis Chamber (Shandon Scientific Company, England), using a regulated power supply. Oxoid barbitone-acetate buffer, ionic

strength 0.025, pH 8.6 was used in the electrophoresis bath. The three soluble antigens used in the immunoelectrophoresis were heat extract, Westphal-type lipopolysaccharide and ultrasonic extract. All these antigens were prepared in the same way as the antigens for the immunodiffusion and the passive haemagglutination tests, except that either the bacterial suspensions being treated or the final preparations were suspended in barbitone-acetate buffer, ionic strength 0.025, pH 8.6. One per cent. Difco Noble agar in the same buffer as used for the preparation of antigens with 0.01 per cent. merthiolate added was used in the test. A standard microscopic slide (25 x 76 mm.) or a photographic plate (80 x 80 mm.) was placed on a horizontal surface and then 2 or 6.5 ml. respectively of the melted agar was poured on, giving a gel layer of about 1 mm. thickness. Wells (1.5 mm. diameter) and troughs (2 mm. width) were cut by the use of a template (Shandon Scientific Co.) to give the distance of 6 mm. between two wells on either side of the trough. The agar was aspirated from the wells with a capillary Pasteur pipette attached to the Venturi pump and the wells were filled with the antigens to be analysed. The slides or plates were then placed in the electrophoresis chamber and strips of chromatography paper (Whatman no. 1) impregnated with the same buffer in the vessels were placed at each end of the plates or slides in order to connect them to the

electrode vessels. A constant current was applied at 100 volts for two hours. After the run of the electrophoresis the agar band was removed from the trough and the trough filled with antiserum. The plate/slide was placed in a large square dish lined with moistened filter paper and stored at 4°C overnight to allow the development of precipitin bands.

(i) Fixation and staining procedures for the clarification of immunoprecipitates

The procedures and reagents employed were similar to those described by Scheidegger (1955). After precipitation all non-precipitated materials were washed out of the agar by immersion of the slide/plate in the same buffer used for the preparation of the agar gel for 24 hours at room temperature. After washing in the buffer the slide/plate was rinsed in deionized water for 5 minutes and the whole surface of the agar layer was covered evenly with a sheet of moistened filter paper (Whatman no. 1). Much care was always taken to avoid air bubbles between the agar layer and filter paper or slide. The slide was then dried overnight in an incubator at 37°C. The filter paper was removed carefully from the dried agar layer after wetting slightly in order to reduce paper residues on the agar surface. The slide was then placed for 5 minutes in a large square dish filled with 2 per cent. acetic acid solution to fix the precipitate.

The fixed slide was transferred to a staining jar containing 0.5 per cent. Amido black in methanol-glacial acetic acid (9:1). After 7 minutes staining the slide was washed in methanol-glacial acetic acid (9:1) until all unbound stain was removed. The slide was then examined for precipitin bands and photographed where necessary.

3. RESULTS

(a) Slide agglutination test

Six strains (RDV55, ERS12, ERS29, NCTC3365, NCTC8529 and NCTC8644) were selected initially for the preparation of antisera. These strains were chosen as representing three haemolytic and three non-haemolytic strains. Two different kinds of antisera were prepared against each of these six strains; one was raised to the heated vaccine and the other to the formolised vaccine. Heated slide antigens were tested against antiserum prepared to the heated vaccine since the formolised cell preparations often showed autoagglutination and the living cultures owing to their viscous nature were unsuitable for use as antigens. Using six antisera raised against the heated vaccine of each immunising strain, slide agglutination tests were set up, all 138 strains in the present series being tested. Fifty-nine strains were agglutinated in the slide test against one or more of these antisera while most others did not respond.

For further antiserum production another five strains (RDV1, RDV28, ERS22, ERS37 and ERS53) were chosen from among the seventy-nine strains which failed to agglutinate with the six antisera. When the five antisera prepared against the heated vaccines of these strains were used to test all the strains in the slide

test, they agglutinated twenty-five strains.

From the fifty-four strains which had failed to react at all in the slide test using the eleven antisera prepared so far, a further four strains (RDV9, ERS20, ERS46 and NCTC8987) were selected for antiserum preparation. Each of these four antisera was then tested against all strains of A. equuli in the present work and a further selection made from those strains which were not agglutinated, in order to prepare other antiserum. Continuing this process a total of 29 antisera were finally produced in rabbits against heated vaccines until all strains with the exception of one (ERS2) were agglutinated in the slide test against one or more of these antisera.

The twenty-nine antisera so prepared were then diluted with PBS until they gave optimal agglutination of the homologous strains. Using these diluted antisera, slide agglutination tests were carried out with the heated slide antigens of the 29 strains of A. equuli against which antisera had been prepared, and the results of these tests are shown in Table 18. Only two strains (RDV55 and ERS29) gave identical slide agglutination patterns with antisera prepared against the twenty-nine strains, suggesting that the strains are extremely diverse in their serological characters. Although the strains were extremely heterogeneous in their serological behaviour, there appeared to be some degree

Table 18 Cross agglutination reactions in slide tests between 29 strains of *A. equuli* and their antisera

Antigen	Antiserum prepared against strains:- RDV1	RDV28	RDV45	RDV62	ERS3a	ERS18	ERS20	ERS22	ERS54	ERS31	ERS37	ERS40	ERS46	ERS47b	ERS53	EQ2	EQ3	EQ7	NCTC3365	NCTC8529	NCTC8644	NCTC8987	ERS12	EQ4	RDV9	RDV64	RDV44	RDV55	ERS29	O group
RDV1	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
RDV28	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
RDV45	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3
RDV62	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4
ERS3a	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5
ERS18	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6
ERS20	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7
ERS22	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	8
ERS54	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	9
ERS31	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10
ERS37	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	11
ERS40	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	12
ERS46	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	13
ERS47b	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	14
ERS53	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15
EQ2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	16
EQ3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	17
EQ7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	18
NCTC3365	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	19
NCTC8529	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	20
NCTC8644	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	21
NCTC8987	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	22
ERS12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	+	23
EQ4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	24
RDV9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	25
RDV64	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	26
RDV44	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	27
RDV55	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	+	28
ERS29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	+	28

of antigenic overlap between some of the strains. This was noted with the five antisera prepared against haemolytic strains, RDV9, ERS12, RDV44, RDV55 and ERS29. Slide agglutination tests using the heated slide antigens of all 138 strains of A. equuli examined in the present work were carried out with the 29 antisera. The results of these tests are presented individually in Appendix B. On the basis of cross slide agglutination reactions as many as 136 of the 138 strains studied could be placed in one of 28 groups and the distribution of these strains among the 28 antigenic groups is shown in Table 21. The two strains which could not be placed in any of the 28 groups were strains ERS2 and ERS55. Strain ERS2 failed to react against any of the 29 antisera while strain ERS55 reacted against no less than 7 different antisera. Each of three antisera prepared from strains ERS20, ERS54 and NCTC3365 agglutinated in the slide test only their homologous antigen, but antisera ERS29 and RDV55 gave positive reactions with as many as 41 strains.

Using eight antisera raised to the formolised and the heated vaccines from each of the strains NCTC3365, NCTC8529, NCTC8644 and ERS12, slide agglutination tests were carried out against three different slide antigen preparations (living, formolised and heated slide antigens) prepared from the four strains against which antisera had been raised. The results obtained with these tests are shown in Table 19. An identical slide

Table 19

Slide agglutination reactions between eight antisera prepared with formolised or heated vaccines of four strains of A. equuli and the living, formolised and heated antigens of those four strains

Agglutinable suspension		Antiserum prepared against							
		formolised vaccine				heated vaccine			
Strain	Preparation	NCTC 3365	NCTC 8529	NCTC 8644	ERS 12	NCTC 3365	NCTC 8529	NCTC 8644	ERS 12
NCTC 3365	Living	+	-	-	-	+	-	-	-
	Formolised	+	-	-	-	+	-	-	-
	Heated	+	-	-	-	+	-	-	-
NCTC 8529	Living	-	+	-	-	-	+	-	-
	Formolised	-	+	-	-	-	+	-	-
	Heated	-	+	-	-	-	+	-	-
NCTC 8644	Living	-	-	+	+	-	-	-	-
	Formolised	-	-	+	+	-	-	-	-
	Heated	-	-	-	-	-	-	+	-
ERS12	Living	-	+	-	+	-	+	-	+
	Formolised	-	+	-	+	-	+	-	+
	Heated	-	-	-	+	-	-	-	+

agglutination pattern against two groups of antisera was obtained with either the living or the formolised antigens prepared from the strains tested, whilst the heated antigens from strain NCTC8644 and ERS12 gave patterns of agglutination quite distinct from those obtained with the corresponding living and formolised antigens. The antiserum raised to NCTC8644 heated vaccine reacted strongly against the homologous heated antigen but failed to agglutinate homologous living and formolised antigens. On the other hand, the antiserum raised to the formolised vaccine of the strain NCTC8644 gave the positive reaction against homologous living and formolised antigens whilst failing to react against homologous heated antigen. Moreover, the antiserum raised to NCTC 8529 formolised vaccine gave positive slide reactions against both the living and the formolised antigens prepared from strain ERS12 but failed to react against the heated antigen from that strain. These results suggested that a heat-labile antigen, presumably envelope(K) antigen, may be present on the living cells or the formolised cells of A. equuli. It is of interest to note that, as with the K antigen of most Enterobacteriaceae, a heat-labile antigen of strain NCTC8644 blocked the agglutination of the somatic(O) antigen.

(b) Tube agglutination test

Heated antigens of the 29 strains of A. equuli to

which antisera had been raised were tested against each antiserum using the conventional tube agglutination test, and the results of these tests are given in Table 20. The results with the tube agglutination tests were in close agreement with those obtained with the slide tests (Table 18). Each antiserum agglutinated its homologous strain to the highest titre which ranged from 1 in 160 to 1 in 2,560. Although the organisms formed an extremely heterogeneous group, there appeared to be a degree of antigenic overlap between some of the strains. This was shown most markedly with the five antisera against strains ERS12, EQ4, RDV44, RDV55 and ERS29. However, the 29 strains so examined could be classified into the 28 groups on the basis of the cross agglutination reactions. The specific antigens of A. equuli concerned in this study are assumed to consist of various factors in the same way as in the O groups of Salmonella. Therefore, the term 'O group' is used for the differentiation of the somatic (O) antigens of A. equuli.

Tube agglutination tests using heated antigens of all 138 strains of A. equuli were carried out with the 29 antisera. The results of these tests are presented in Appendix C. On the basis of these cross agglutination reactions, all the strains with the exception of two (ERS2 and ERS55) could be arranged in one of the 28 O-groups and the distribution of these strains among the 28 antigenic groups is shown in Table 21. These

Table 20 Cross agglutination reactions in tube test between 29 strains of A. equuli and their antisera

Antigen	Antiserum prepared against strain:- RDV1	RDV28	RDV45	RDV62	ERS3a	ERS18	ERS20	ERS22	ERS54	ERS31	ERS37	ERS40	ERS46	ERS47b	ERS53	EQ2	EQ3	EQ7	NCTC3365	NCTC8529	NCTC8644	NCTC8987	ERS12	EQ4	RDV9	RDV64	RDV44	RDV55	ERS29	O group
RDV1	320	0	0	0	0	0	0	0	20	0	20	0	20	0	20	0	0	0	0	0	0	0	0	0	40	0	0	20	20	1
RDV28	0	640	40	0	0	0	0	0	0	0	20	0	0	0	0	40	0	0	0	0	20	0	0	0	0	0	0	0	0	2
RDV45	0	0	640	0	0	0	0	0	0	0	20	0	20	0	0	20	0	0	0	0	20	0	0	0	20	0	0	0	0	3
RDV62	0	0	0	640	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
ERS3a	0	0	0	0	640	0	0	0	40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	20	0	0	0	0	0	5
ERS18	0	0	0	0	0	640	0	0	0	0	0	0	0	80	40	0	0	0	0	0	0	0	0	0	20	0	0	0	0	6
ERS20	0	0	20	0	0	0	320	0	0	0	80	0	20	0	0	20	0	0	0	0	0	0	0	0	0	0	0	0	0	7
ERS22	0	0	0	0	0	0	0	640	0	0	20	0	0	0	0	0	0	0	0	0	0	0	0	0	20	0	0	0	0	8
ERS54	0	0	0	0	40	0	0	160	320	0	0	0	0	0	0	0	0	0	0	0	0	0	0	40	0	0	0	0	0	9
ERS31	0	0	0	0	0	0	0	0	20	640	0	0	0	0	0	0	0	0	0	0	0	0	0	20	0	0	0	0	0	10
ERS37	0	0	0	0	0	20	0	0	0	0	320	0	0	0	0	20	0	0	0	0	0	0	0	0	0	0	0	0	0	11
ERS40	0	0	0	0	0	0	0	0	0	40	0	640	40	0	0	40	0	0	0	0	20	20	0	40	20	0	0	0	0	12
ERS46	0	0	0	0	0	0	0	0	0	0	0	0	320	0	0	0	0	0	0	0	0	0	0	0	20	0	0	0	0	13
ERS47b	0	0	0	0	0	0	0	0	0	0	0	0	0	640	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14
ERS53	0	0	0	0	20	0	0	0	0	0	0	0	0	0	640	20	0	0	0	0	0	0	0	0	20	0	0	0	0	15
EQ2	0	0	0	0	0	0	0	0	20	0	0	0	0	40	0	160	0	0	20	0	0	0	0	40	20	0	0	0	0	16
EQ3	0	0	0	0	0	0	0	0	0	0	20	0	0	0	0	0	160	0	0	0	0	0	0	20	0	0	0	0	0	17
EQ7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	640	0	0	0	0	0	0	0	0	0	0	0	18
NCTC3365	0	20	40	0	0	20	20	0	20	0	20	0	20	0	20	40	0	0	640	0	20	40	20	40	40	80	0	20	20	19
NCTC8529	0	0	0	0	0	0	0	0	20	0	0	0	0	0	0	0	0	0	0	640	0	0	0	20	0	0	0	0	0	20
NCTC8644	20	0	0	0	0	0	0	0	0	0	20	0	0	0	0	0	0	0	0	0	640	0	0	0	0	0	0	0	0	21
NCTC8987	0	0	0	0	0	0	0	0	0	0	20	0	0	0	0	0	0	0	0	0	0	320	0	0	20	0	0	0	0	22
ERS12	0	20	0	0	0	0	0	0	0	0	40	0	40	0	0	0	40	0	0	0	20	20	1280	320	40	20	20	80	80	23
EQ4	0	0	0	0	20	20	0	40	20	20	0	40	0	0	0	20	0	0	0	0	0	0	160	640	0	0	20	40	160	24
RDV9	0	0	0	0	0	0	0	0	0	40	0	0	20	40	0	0	0	0	0	0	0	0	20	80	640	40	0	20	20	25
RDV64	0	0	0	0	0	0	0	0	0	0	20	0	0	0	0	0	0	0	0	0	0	40	0	40	40	320	0	20	20	26
RDV44	0	0	0	0	0	0	0	0	0	20	20	0	0	0	0	0	0	0	0	0	0	0	20	0	0	0	640	40	160	27
RDV55	0	80	20	0	20	20	0	20	20	0	80	0	20	0	20	40	0	0	0	0	20	20	80	80	20	20	40	640	2560	28
ERS29	0	40	40	0	0	0	0	0	0	0	40	0	20	0	20	40	0	0	0	0	40	40	160	80	20	20	160	640	2560	28

O indicate no agglutination at a dilution of 1 in 20.

Table 21

Distribution of 28 O groups among 138 strains of *A. equuli*

O group	Group antiserum raised to strain	No. of strains from		Total
		Horse	Pig	
1	RDV1	6	0	6
2	RDV28	5	0	5
3	RDV45	3	0	3
4	RDV62	3	0	3
5	ERS3a	2	0	2
6	ERS18	2	0	2
7	ERS20	1	0	1
8	ERS22	4	0	4
9	ERS54	1	0	1
10	ERS31	2	0	2
11	ERS37	4	0	4
12	ERS40	3	0	3
13	ERS46	8	0	8
14	ERS47b	7	0	7
15	ERS53	5	2	7
16	EQ2	4	0	4
17	EQ3	1	3	4
18	EQ7	4	0	4
19	NCTC3365	1	0	1
20	NCTC8529	5	0	5
21	NCTC8644	5	0	5
22	NCTC8987	2	0	2
23	ERS12	12	0	12
24	EQ4	1	0	1
25	RDV9	2	0	2
26	RDV64	3	0	3
27	RDV44	10	0	10
28	ERS29	20	5	25
Ungroupable		2	0	2
Total		128	10	138

results agreed closely with those obtained with the slide tests which were used mainly for 'screening' purposes. Four antisera raised to strains ERS20, ERS54, NCTC3365 and EQ4 agglutinated no other organisms to a titre comparable with that obtained with the homologous antigen, while the remaining 25 antisera agglutinated others and enabled one to classify the organisms into one of the other 24 O-groups. In order to investigate the antigenic structure and relationship of strains falling into the same O groups on the basis of their cross agglutination reactions, agglutinin-absorption tests were performed with each strain which showed cross agglutination with an antiserum at a titre comparable with that observed with the homologous strain. The results of these tests are presented in Table 22. There was complete absorption of antibodies when the antisera of O groups 2, 3, 4, 5, 6, 10, 11, 12, 14, 16, 18, 20, 22, 25 and 26 were absorbed with the organisms of the corresponding O group, suggesting the complete homogeneity of the organisms classified into those O groups. Some of the strains belonging to the O groups 1, 8, 13, 15, 17, 21, 23, 27 and 28 failed to exhaust the antibodies in their group antiserum but most of the strains of these groups did absorb the specific antibodies from their O group antiserum.

Antisera ERS12 (O group 23), RDV44 (O group 27), RDV55 (O group 28) and ERS29 (O group 28) were absorbed

Table 22

Agglutination titre of 24 antisera before and after absorption with heated antigens prepared from each strain of the same antigenic group

Antiserum against strain		Agglutinable and absorbing antigens prepared with strain:-																
		RDV1	RDV2	RDV3	RDV5	ERS1	ERS14	RDV28	RDV29	RDV30	RDV31	RDV32	RDV45	ERS49a	ERS49b	RDV61	RDV62	RDV63
RDV1 (Group 1)	a *	320	320	320	320	320	320	...	...	...	...	...	...	...	...	...	...	...
	b		0	0	0	40	0	...	...	...	...	...	...	...	...	...	...	...
RDV28 (Group 2)	a	...	...	...	...	...	...	640	640	640	640	640	...	...	...	...	...	...
	b	...	...	...	...	...	...		0	0	0	0	...	...	...	...	...	...
RDV45 (Group 3)	a	...	...	...	...	...	...	...	...	...	...	...	640	320	320	...	...	...
	b	...	...	...	...	...	...	...	...	...	...	...		0	0	...	...	...
RDV62 (Group 4)	a	...	...	...	...	...	...	...	...	...	...	...	...	...	...	320	640	320
	b	...	...	...	...	...	...	...	...	...	...	...	...	...	...		0	0

Table 22 continued

Antiserum against strain		Agglutinable and absorbing antigens prepared with strain:-													
		ERS3a	ERS3b	ERS18	ERS19	ERS22	ERS21	RDV52	RDV53	ERS31	ERS32	ERS37	RDV33	ERS4	ERS5
ERS3a (Group 5)	a *	640	640	...	...	...	...	...	...	...	...	...	...	...	...
	b		0	...	...	...	...	...	...	...	...	...	...	...	...
ERS18 (Group 6)	a	...	...	640	320	...	...	...	...	...	...	...	...	...	...
	b	...	...		0	...	...	...	...	...	...	...	...	...	...
ERS22 (Group 8)	a	...	...	...	...	320	320	160	160	...	...	...	...	...	...
	b	...	...	...	...		0	80	40	...	...	...	...	...	...
ERS31 (Group 10)	a	...	...	...	...	...	...	...	...	640	640	...	...	...	...
	b	...	...	...	...	...	...	...	...		0	...	...	...	...
ERS37 (Group 11)	a	...	...	...	...	...	...	...	...	...	...	320	160	320	320
	b	...	...	...	...	...	...	...	...	...	...		0	0	0

Table 22 continued

Antiserum against strain		Agglutinable and absorbing antigens prepared with strain:-																	
		ERS40	ERS17	ERS39	ERS46	ERS35	ERS36	ERS42	ERS43	ERS45	RDV46	RDV21	ERS47b	ERS47a	RDV20	ERS16	ERS41	ERS44	ERS52
ERS40 (Group 12)	a *	640	640	640	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
	b		0	0	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
ERS46 (Group 13)	a	...	...	...	320	320	160	320	160	160	160	320	...	...	...	...	...	...	...
	b	...	...	...		0	40	0	0	40	80	0	...	...	...	...	...	...	...
ERS47b (Group 14)	a	...	...	...	...	...	...	...	...	...	...	...	640	320	320	320	320	320	640
	b	...	...	...	...	...	...	...	...	...	...	...		0	0	0	0	0	0

Table 22 continued

Antiserum against strain		Agglutinable and absorbing antigens prepared with strain:-														
		ERS53	ERS24	ERS25	ERS27	ERS30	EQ5	EQ8	EQ2	ERS56	RDV11	RDV15	EQ3	ERS26	EQ1	EQ6
ERS53 (Group 15)	a *	640	320	320	320	320	160	160	...	...	...	...	...	...	...	...
	b		0	0	0	0	320	320	...	...	...	...	...	...	...	...
EQ2 (Group 16)	a	...	...	...	...	...	...	...	160	160	160	160	...	...	...	...
	b	...	...	...	...	...	...	...		0	0	0	...	...	...	...
EQ3 (Group 17)	a	...	...	...	...	...	...	...	...	...	...	...	160	80	80	160
	b	...	...	...	...	...	...	...	...	...	...	...		20	0	0

Table 22 continued

Antiserum against strain		Agglutinable and absorbing antigens prepared with strain:-															
		EQ7	ERS48a	ERS48b	RDV6	NCTC 8529	RDV34	ERS23	ERS33	ERS34	NCTC 8644	NCTC 8794	NCTC 9435	RDV60	ERS51	NCTC 8987	ERS11
EQ7 (Group 18)	a *	640	320	160	320	...	...	...	...	...	...	...	...	...	...	...	...
	b		0	0	0	...	...	...	...	...	...	...	...	...	...	...	...
NCTC8529 (Group 20)	a	...	...	...	...	640	640	320	640	640	...	...	....	...	...	...	...
	b	...	...	...	...		0	0	0	0	...	...	...	...	...	...	...
NCTC8644 (Group 21)	a	...	...	...	...	...	...	...	...	...	1280	160	1280	160	160	...	...
	b	...	...	...	...	...	...	...	...	...		320	0	320	640	...	...
NCTC8987 (Group 22)	a	...	...	...	...	...	...	...	...	...	...	...	...	...	...	320	320
	b	...	...	...	...	...	...	...	...	...	...	...	...	...	...		0

Table 22 continued

Antiserum against strain		Agglutinable and absorbing antigens prepared with strain:-													
		ERS12	RDV14	RDV22-25	RDV26	RDV27	RDV59	ERS7	ERS10	ERS13	RDV9	RDV10	RDV64	RDV4	RDV65
ERS12 (Group 23)	a *	1280	1280	640	640	640	640	640	640	640	...	...	...	...	...
	b		80	0	20	0	160	0	0	0	...	...	...	...	...
RDV9 (Group 25)	a	...	...	...	...	...	...	...	...	...	640	640	...	...	...
	b	...	...	...	...	...	...	...	...	...		0	...	...	...
RDV64 (Group 26)	a	...	...	...	...	...	...	...	...	...	...	...	640	320	320
	b	...	...	...	...	...	...	...	...	...	...	...		0	0

Table 22 continued

Antiserum against strain		Agglutinable and absorbing antigens prepared with strain:-																
		RDV44	RDV35-37	RDV42-43	RDV56	ERS28 & 38	EQ14	ERS29	RDV7-8	RDV12-13	RDV16-19	RDV38-41	RDV50-51	RDV54-55	ERS6	ERS8 & 15	EQ9	EQ10-13
RDV44 (Group 27)	a *	640	640	320	640	320	640	...	...	...	...	...	...	...	...	...	...	...
	b		0	0	0	160	0	...	...	...	...	...	...	...	...	...	...	...
ERS29 (Group 28)	a	...	...	...	...	...	...	2560	2560	1280	1280	1280	1280	2560	1280	2560	2560	2560
	b	...	...	...	...	...	...		0	80	80	80	160	0	160	0	160	80

* a = reciprocal titre before absorption with strain indicated
b = reciprocal titre with homologous organism after absorption with strain indicated
0 = no agglutination at a dilution of 1 in 20
... = not tested

Table 23

Agglutination reactions with antisera ERS12, ERS29, RDV44 and RDV55 before and after absorption with the heated antigens of strains of A. equuli agglutinated in slide tests

Antiserum against strain		Agglutinable and absorbing antigens prepared with strain:-				
		EQ4	ERS12	RDV44	RDV55	ERS29
ERS12	a*	320	1280	20	80	80
	b	1280		1280	1280	1280
RDV44	a	20	20	640	40	80
	b	640	640		320	320
RDV55	a	40	80	40	640	640
	b	640	640	320		0
ERS29	a	160	320	160	2560	2560
	b	2560	1280	1280	0	

* a = reciprocal titre with strain indicated

b = reciprocal titre with homologous antigen after absorption with strain indicated

0 = no agglutination at a dilution of 1 in 20

with those strains of A. equuli which they had cross-agglutinated in the slide test. The results with these tests are presented in Table 23.

Antiserum ERS12 was absorbed with four such strains and in every case the titre of the antiserum for the homologous antigen remained at the same level as that of the unabsorbed antiserum, even though antibodies against the absorbing strains had been completely exhausted. Similar results were also obtained with the other three antisera.

(c) Passive haemagglutination test

The passive haemagglutination (HA) test in various modifications has been extensively applied to the determination of antigens and to the identification of microorganisms, and has been found to be a sensitive procedure for the antigenic analysis and for the serological typing of strains (Carter, 1955 and 1972; Biberstein, Gills and Knight, 1960; Kim and Lee, 1973). The present work was undertaken to investigate the feasibility of applying the passive HA test for the determination of anti-A. equuli antibodies in rabbit and for the serological typing of the strains of this species of Actinobacillus.

(i) Standardisation of the passive haemagglutination test components for the demonstration of antibody to A. equuli

a) Erythrocyte-modifying antigens of A. equuli

First studied was the question whether A. equuli antigens modify erythrocytes of sheep, thus rendering them agglutinable by homologous antiserum. Heat extracts of strains NCTC8529 and NCTC8644 were used to modify sheep washed erythrocytes which were then used as a 1 per cent. suspension in the passive HA test, the results of which are shown in Table 24. It is evident from the results that the antisera agglutinate to the highest titre the erythrocytes treated with homologous heat extract and only in relatively low titre those treated with heterologous antigens. These results are largely in agreement with those obtained with agglutination test.

Table 24

The results of the haemagglutination tests of 2 strains of A. equuli NCTC8529 and NCTC8644 with their antisera raised to heated vaccine

Antigen to strain	Antiserum to strain	Reciprocal antiserum dilution						
		80	160	640	2560	5120	10240	20480
NCTC 8529	NCTC8529	4	4	4	4	4	2	0
	NCTC8644	4	1	0	0	0	0	0
NCTC 8644	NCTC8529	4	2	0	0	0	0	0
	NCTC8644	4	4	4	4	4	4	3

Figures in the table indicate the degree of haemagglutination (see p.128).

As the bacterial antigens that can be absorbed on to erythrocytes are the soluble components liberated from bacteria by various treatments, four soluble antigenic preparations, viz. 2.5 per cent. saline extract, ultrasonic extract, heat extract and Westphal-type lipopolysaccharide, were tested to determine their ability to adsorb on the surface of erythrocytes and their agglutinability in the presence of homologous antiserum. Several strains were investigated in this way and the results with one strain, NCTC8529, are shown in Table 25. These show that the heat extract and the Westphal-type lipopolysaccharide were the most satisfactory agents, while the ultrasonic extract was less effective for the modification of erythrocytes. The saline extract failed altogether to modify the erythrocytes.

Table 25

The effect of different erythrocyte-modifying antigen preparations of *A. equuli* strain NCTC8529 in relation to the sensitivity of the haemagglutination test.

Antigen preparation	Titres of antiserum to NCTC8529 formolised vaccine with its homologous antigen								
	80	160	320	640	1280	2560	5120	10240	20480
Saline extract	0	0	0	0	0	0	0	0	0
Heat extract	4	4	4	4	4	4	4	2	0
Westphal-type LPS*	4	4	4	4	4	4	4	3	0
Ultrasonic extract	4	4	4	4	3	2	0	0	0

*LPS = lipopolysaccharide

Figures in the table indicate the degree of haemagglutination.

Since the heat-stable soluble antigens appeared to be the most effective agent for the modification of erythrocytes the following experiments were carried out to determine the effects of temperature and time on the extraction of erythrocyte-modifying antigens. Ten per cent. bacterial suspensions in PBS were treated at various temperatures and for various times and were then centrifuged to remove the cells. The supernatants so obtained were used to modify erythrocytes for the passive HA test. Several strains of A. equuli were used in this experiment and the results with one NCTC8644, are shown in Table 26.

This table shows that the soluble antigens extracted from the bacterial suspensions by boiling or autoclaving modify the erythrocytes most effectively, thus resulting in specific haemagglutination with the homologous antiserum raised to heated vaccine in as high a dilution as 1 in 10240. Good results were obtained with the antigens treated at 56°C or 70°C for 2 hours. However, the antigens extracted at 4°C or room temperature for twenty hours failed to modify the erythrocytes.

To determine what effect the concentration of the antigen has upon the passive haemagglutination reaction, heat extracts (100°C for 2 hours) from cell suspensions of strains NCTC8529 and NCTC8644 in four concentrations (2.5, 5, 10 and 20 per cent.) were each tested with serial dilutions of the homologous antiserum raised to

Table 26

The effect of temperature and time on the extraction of erythrocyte-modifying antigens of A. equuli strain NCTC8644

Temperature for extraction (C)	Time for extraction (hour)	Highest antiserum dilution giving haemagglutination	
		Formolised vaccine	Heated vaccine
4	20	0	0
RT*	20	0	0
37	20	40(1)	640(3)
56	2	80(1)	1280(3)
70	2	160(2)	1280(3)
100	1	1280(2)	5120(4)
100	2	1280(3)	10240(3)
100	4	2560(1)	10240(3)
121	1/4	2560(2)	10240(3)

* RT = room temperature (18-22°C)

0 indicates no haemagglutination at a dilution of 1 in 40.

Figures in parentheses indicate the degree of haemagglutination.

heated vaccine, and the results with these tests are shown in Table 27.

Table 27

The effect of bacterial concentrations on the extraction of the erythrocyte-modifying antigens of A. equuli

Bacterial concentration (%)	Highest dilution of antiserum showing haemagglutination	
	NCTC8529	NCTC8644
2.5	5120(2)	5120(4)
5	5120(3)	10240(3)
10	10240(3)	10240(4)
20	Not tested	10240(4)

Figures in parentheses indicate the degree of haemagglutination.

There was no marked difference between the antigen preparations. However, complete haemagglutination was obtained at highest serum dilutions when antigens extracted from a 10 and 20 per cent. bacterial suspensions were used for erythrocyte modification. Although satisfactory sensitisation of erythrocytes could be obtained with a wide range of antigen concentration, the most effective agent for the modification of erythrocytes appeared to be the heat extract (100°C for 2 hours) from a 10 per cent. suspension in PBS and this, therefore, was

employed in all the subsequent work.

Attempts were made to modify successive batches of erythrocytes using the supernatant from each sensitisation for the next batch of erythrocytes. The modifying capacity of the antigen appeared to be progressively reduced as shown in Table 28, but the fact that the fourth batches of the erythrocytes so modified were agglutinated by a 1 in 10240 dilution of their homologous antisera suggested that a single antigen solution could be repeatedly used to coat batches of erythrocytes without losing their erythrocyte-modifying activity.

Table 28

Haemagglutination of batches of erythrocytes treated successively with a single dose of heat extract

Batch of red cells in order of treatment	Highest dilution of antiserum showing haemagglutination	
	NCTC8529	NCTC8644
1	20480(4)	20480(4)
2	20480(3)	20480(4)
3	10240(4)	20480(4)
4	10240(4)	20480(2)
5	5120(4)	10240(4)
6	5120(3)	10240(4)
7	2560(3)	10240(4)

Figures in parentheses indicate the degree of haemagglutination.

Table 29

The effect of heat on erythrocyte-modifying activity of the ultrasonic extract of A. equuli

Temperature (C)	Time (hour)	Highest dilution of antiserum showing haemagglutination	
		NCTC8529	NCTC8644
Untreated		2560(4)	5120(2)
56	1	10240(3)	10240(2)
70	1	10240(3)	10240(3)
100	1	20480(4)	20480(4)
100	2	20480(4)	20480(4)
121	1/4	20480(1)	20480(4)

Figures in parentheses indicate the degree of haemagglutination.

b) The effect of heat on the erythrocyte-modifying capacities of ultrasonic extracts of A. equuli

To determine the effect of heat on the erythrocyte-modifying properties of the ultrasonic extract of A. equuli, the following experiment was performed. Ultrasonic extracts of strains NCTC8529 and NCTC8644 were prepared and then heated at various temperatures for different periods of time, viz. 56°C for one hour, 70°C for one hour, 100°C for one hour and two hours and 121°C for 15 minutes. On centrifugation quite a large deposit of material rendered insoluble during heating was obtained. The preparations so obtained were used to modify erythrocytes which were then employed in the passive HA test. As shown in Table 29, heating, in general, appeared to enhance the erythrocyte-modifying capacity of ultrasonic extracts of A. equuli, this being shown most notedly with the antigens treated at 100°C.

c) The effect of various agents on the erythrocyte-modifying capacities of ultrasonic extracts of A. equuli

Ultrasonic extracts of 10 per cent. cell suspensions of strains NCTC8644 and ERS29 were treated with trypsin, pepsin, acid, alkali and phenol to determine the effect of these agents on the erythrocyte-modifying

activity of the soluble antigens of A. equuli.

i) Trypsin treatment

The procedure employed was a minor modification of the method described by Prince and Smith (1966). One volume of the ultrasonic extract was treated with an equal volume of trypsin (Difco, 1:250) solution (0.75 mg. trypsin per ml. of M/15 phosphate buffer, pH 7.6) for two and a half hours in a water bath at 37°C. On centrifugation at 2,200 x G for 30 minutes, a small amount of deposit rendered insoluble during the trypsinisation was obtained. The supernatant was dialysed against PBS for 48 hours to restore the tonicity and to inactivate the trypsin.

ii) Pepsin treatment

The conditions of pepsin treatment were mainly those used by Prince and Smith (1966). To one volume of the ultrasonic extract an equal volume of pepsin (B.D.H.) solution (0.75 mg. pepsin per ml. of 0.03N HCl) was added and the mixtures were incubated for one hour at 37°C. A considerable amount of deposit separated on centrifugation at 2,200 x G for 30 minutes. The supernatant so obtained was dialysed against PBS for 48 hours at room temperature to adjust the pH and to restore the tonicity of the solution.

iii) Acid treatment

The ultrasonic extract was treated with an equal volume of 0.03N HCl for one hour in a water

bath at 37°C and then centrifuged to discard the insoluble materials. The supernatant was dialysed against PBS for 48 hours to restore the reaction and the tonicity.

iv) Alkali treatment

One portion of the ultrasonic extract was added to an equal volume of 0.02N NaOH and placed in a water bath at 37°C for 2 hours according to the method described by Davies et al. (1958) and then centrifuged. The supernatant was dialysed against PBS for 48 hours.

v) Phenol treatment

One portion of the ultrasonic extract was treated with an equal volume of 90 per cent. phenol solution by the same method for the preparation of the Westphal-type lipopolysaccharides.

The results of the passive HA tests using the treated ultrasonic extract are shown in Table 30. Phenol and weak alkali treatment enhanced the erythrocyte-modifying activities of the ultrasonic extract considerably. A similar result was obtained with the ultrasonic extract treated with trypsin. In contrast, however, a fall in titre was observed with the pepsin-treated ultrasonic extract, but no very marked change in the haemagglutination titre was obtained with the acid-treated ultrasonic extract.

Table 30

The effects of various agents on the erythrocyte-modifying activity of the ultrasonic extract of A. equuli

Ultrasonic extract treated with	Highest dilution of antiserum showing haemagglutination	
	NCTC8644	ERS29
Untreated	5120(2)	5120(3)
Trypsin	20480(4)	10240(4)
Pepsin	1280(2)	<80
0.03N HCl	5120(4)	2560(3)
0.02N NaOH	10240(4)	40960(3)
Phenol	20480(4)	40960(4)

Figures in parentheses indicate the degree of haemagglutination

d) Optimum concentration of antigen-treated erythrocytes in the passive haemagglutination test for A. equuli

To determine the optimum concentration of antigen treated erythrocytes in the passive HA test for the demonstration of antibodies of A. equuli, seven different concentrations of erythrocytes modified with heat extract of strain NCTC8529 were tested with serial dilutions of the homologous antiserum. The results given in Table 31 show that the highest dilution of

antiserum to show positive haemagglutination was obtained when 0.5 and 0.25 per cent. suspensions of antigen-treated erythrocytes were used in the test. Although a greater sensitivity was obtained using more dilute erythrocyte suspensions, for practical purposes the minimum quantity of erythrocytes necessary to produce a reaction which is easily visible limits the dilutions of the erythrocyte suspension that might be employed. The optimum concentration was considered to be 0.5 per cent.

Table 31

Variation in endpoint of haemagglutination in relation to antigen-treated erythrocyte concentration

Modified erythrocyte concentration (%)	Reciprocal dilution of antiserum NCTC8529							
	160	320	640	1280	2560	5120	10240	20480
2.0	4	4	4	4	1	0	0	0
1.5	4	4	4	4	1	0	0	0
1.0	4	4	4	4	4	1	0	0
0.75	4	4	4	4	4	2	0	0
0.5	4	4	4	4	4	4	3	0
0.25	4	4	4	4	4	4	3	0
0.01	quantity of cells insufficient							

Figures in the table indicate the degree of haemagglutination.

e) Haemagglutination with different species of erythrocytes modified with heat extract of A. equuli

It was considered of interest to determine whether the erythrocytes of other species also could be modified. Washed erythrocytes of ox, horse chicken and guinea-pig were utilised in addition to those from sheep. The results of a representative experiment with A. equuli ERS12 heat extract are presented in Table 32 from which it can be seen that the erythrocytes from all the species used in the experiment adsorbed the antigen, and thus become agglutinable by the homologous antiserum. The titre of antiserum with the erythrocytes of ox, horse, chicken and sheep was roughly identical while considerably low titre was obtained with the guinea-pig erythrocytes.

Table 32

Variations of the haemagglutination titres in relation to the species of erythrocyte

Species of cells	Reciprocal dilution of antiserum ERS12							
	320	640	1280	2560	5120	10240	20480	40960
Chicken	4	4	4	4	4	2	0	0
G. pig	4	4	3	0	0	0	0	0
Horse	4	4	4	4	4	4	3	0
Ox	4	4	4	4	4	2	0	0
Sheep	4	4	4	4	4	4	3	0

Figures in the table indicate the degree of haemagglutination

(ii) Serogrouping of A. equuli by means of the passive haemagglutination test

To determine the O group specificity of the strains of A. equuli using the passive HA test described above, cross haemagglutination tests with the 28 O-group antisera were carried out against sheep erythrocytes modified with heat extract of each of the 28 strains of A. equuli against which antisera had been raised. The results of these tests are presented in Table 33. In all cases the antiserum agglutinated to the highest titre erythrocytes treated with homologous heat extract and only in relatively low titre those treated with heterologous antigens. These results largely agreed with those of the agglutination test. However, it is interesting to note that the titres of all antisera in the passive HA test were considerably higher than those in the agglutination test and, moreover, the results of the passive HA test were much more easily interpreted than those of conventional agglutination tests, indicating that this method is more sensitive than the agglutination test for the determination of the antigenic structure of A. equuli.

To investigate the feasibility of applying the polyvalent haemagglutination test described by Neter (1956) and Neter, Gorzinski, Gino, Westphal and Lüderitz (1956c) for the detection of any homologous antibody to A. equuli, the following experiments were

Table 33 Cross passive haemagglutination reactions between the 28 O-groups of A. equuli and their antisera

Antigen	Antiserum prepared against the heated vaccine to strain:-																												
	RDV1	RDV28	RDV45	RDV62	ERS3a	ERS18	ERS20	ERS22	ERS54	ERS31	ERS37	ERS40	ERS46	ERS47b	ERS53	EQ2	EQ3	EQ7	NCTC3365	NCTC8529	NCTC8644	NCTC8987	ERS12	EQ4	RDV9	RDV64	RDV44	ERS29	O group
RDV1	**2560**	0	0	0	40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
RDV28	0	**10240**	0	0	0	0	0	0	0	0	80	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
RDV45	0	0	**5120**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
RDV62	0	0	0	**5120**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
ERS3a	40	40	40	40	**20480**	0	0	0	640	0	0	0	80	40	0	0	0	0	0	0	0	0	0	80	0	0	0	0	5
ERS18	0	0	0	0	0	**5120**	40	0	0	0	0	0	0	0	0	0	40	0	0	40	0	0	0	0	40	0	0	40	6
ERS20	0	0	0	0	0	0	**1280**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7
ERS22	0	0	0	0	160	0	0	**10240**	160	40	0	0	0	40	0	0	0	40	0	0	0	0	0	0	0	0	80	0	8
ERS54	0	0	0	0	1280	0	40	1280	**5120**	0	0	0	0	0	0	80	0	40	40	0	0	0	0	0	80	0	0	0	9
ERS31	0	0	0	0	0	0	0	0	0	**10240**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10
ERS37	0	0	0	0	0	0	0	0	0	0	**1280**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	11
ERS40	0	0	40	80	0	40	0	0	0	0	0	**5120**	0	0	0	0	0	0	0	0	0	0	40	0	0	0	0	0	12
ERS46	0	0	0	40	640	80	40	0	80	80	0	0	**20480**	40	40	40	0	80	0	0	40	0	0	80	40	0	0	0	13
ERS47b	0	80	0	80	40	80	80	80	40	40	40	0	0	**10240**	0	80	80	80	80	0	80	0	0	80	80	40	0	40	14
ERS53	0	0	0	40	0	80	0	0	0	0	40	0	0	0	**20480**	0	0	0	0	0	0	0	0	0	0	0	0	0	15
EQ2	0	0	40	160	0	0	80	0	0	40	0	160	0	40	40	**5120**	40	160	160	0	160	640	0	640	160	160	40	80	16
EQ3	0	0	40	80	0	0	80	0	0	40	80	40	0	0	40	80	**2560**	80	80	0	160	640	0	640	80	80	40	80	17
EQ7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**40960**	0	0	40	0	0	0	0	0	0	0	18
NCTC3365	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**5120**	0	0	0	0	0	0	0	0	0	19
NCTC8529	320	0	80	160	640	320	160	0	1280	160	160	80	160	160	80	160	80	320	320	**10240**	160	320	640	640	160	320	80	80	20
NCTC8644	80	80	40	80	320	320	80	0	160	160	40	80	80	0	40	40	0	160	80	80	**20480**	0	0	80	80	80	80	80	21
NCTC8987	0	0	0	0	80	0	0	0	0	40	0	0	0	0	0	40	0	0	80	0	40	**20480**	0	0	40	40	0	40	22
ERS12	40	40	0	0	1280	80	0	0	0	40	40	0	40	80	40	0	0	40	40	160	40	0	**20480**	1280	640	160	160	640	23
EQ4	0	0	0	40	0	0	40	0	0	0	0	40	40	0	0	0	0	0	0	0	0	0	160	**20480**	1280	40	40	160	24
RDV9	40	80	80	80	0	0	80	0	0	0	80	80	40	0	40	40	80	80	80	40	80	160	80	1280	**20480**	160	40	40	25
RDV64	0	0	40	80	0	40	0	0	0	0	0	40	0	80	0	0	80	40	0	0	0	80	0	80	80	**2560**	40	40	26
RDV44	0	40	0	0	0	0	40	0	0	0	40	0	0	0	0	0	0	0	0	40	0	0	5120	1280	80	160	**40960**	1280	27
ERS29	0	0	0	0	320	160	0	40	160	160	40	40	40	40	0	40	0	80	40	40	80	0	320	80	80	160	40	**20480**	28

O indicate no haemagglutination at a dilution of 1 in 40.

carried out. Erythrocytes modified either individually or simultaneously with each of six heat extracts derived from the different O groups of A. equuli were added to appropriate dilutions of the homologous antisera, and the tests were incubated and read in the usual manner. The results of these experiments are shown in Table 34. It is apparent that erythrocytes modified with either one or as many as six heat extracts of A. equuli appeared to be equally convenient positive carriers of antigen for the demonstration of the respective antibodies.

Table 34

Comparison of serological reactivity of erythrocytes treated individually or simultaneously with six different heat extracts of A. equuli

Antiserum and antigen to strain	Highest dilution of antisera which agglutinated erythrocytes treated with:-	
	Homologous antigen alone	Simultaneously all 6 antigens
ERS53	20480(3)	10240(2)
EQ3	2560(4)	1280(4)
NCTC8529	10240(3)	10240(3)
NCTC8644	20480(3)	20480(3)
ERS12	10240(4)	10240(4)
RDV9	20480(3)	10240(3)

Figures in parentheses indicate the degree of haemagglutination.

(iii) Haemagglutination-inhibition reaction

To evaluate the antigenic relationships of two strains of A. equuli, RDV55 and ERS29, which showed marked cross reactions both in the agglutination test and in the passive HA test, the haemagglutination-inhibition test was employed. Table 35 shows the results of these tests. The inhibitory titres of antigen RDV55 and ERS29 against antiserum RDV55 were both 160 and those against antiserum ERS29 were also 160.

Table 35

Cross haemagglutination-inhibition by the heat extract of A. equuli

Antiserum & antigen to strain	Inhibiting antigen to strain	Reciprocal dilution of antigen						
		40	80	160	320	640	1280	2560
RDV55	RDV55	0	0	0	4	4	4	4
	ERS29	0	0	0	3	4	4	4
ERS29	RDV55	0	0	0	2	3	4	4
	ERS29	0	0	0	2	3	4	4

Figures in the table indicate the degree of haemagglutination.

(d) Immunodiffusion precipitin test

(i) Serological comparison of antigens

The various antigenic extracts obtained from

10 per cent. wet cell suspensions of the strains NCTC8644 and ERS29 were evaluated in agar gel, using their homologous antisera raised both to heated and formolised vaccines and the results are presented in Plates 9 and 10. Strong reactions were obtained with the heat extract (HE), the Westphal-type lipopolysaccharide (LPS) and the sodium desoxycholate extract (SDE) against the homologous antiserum prepared against the heated vaccine, while very weak reactions were observed with all extracts used against the antiserum raised to the formolised vaccine. In view of these results, antisera raised to heated vaccines would be expected to react better with heat stable antigens such as LPS and HE. Lines of identity were observed between HE and the ultrasonic extract (USE), and this was also noted between antigen LPS and SDE. Although LPS and SDE antigens appeared to have at least one more antigenic determinant than the HE, all six antigens gave intense homologous reactions (Plate 11) against the antiserum raised to the heated vaccine. The noticeable spur with antigens LPS and HE indicates the presence of an additional antigenic determinant on antigen LPS that is not present in antigen HE.

Since the differences in the immunodiffusion precipitin test could have been brought about either by the heat treatment or by the chemical agents which had been employed in the extractions, it was decided to

compare the reactivity of the antigens of HE before and after treatment with phenol, and of the LPS, SDE, USE, 2.5% saline extract (SE) and alcohol extract (AE) before and after treatment with heat. All the soluble antigen preparations except the HE were treated with heat at 100°C for 2 hours while antigen HE was treated with phenol in the same way as the LPS was prepared. Treatment of the LPS with heat appeared to have increased the diffusion rate of the additional antigenic determinant(s) on LPS that are not found in the HE antigen (Plate 11). This pattern was similar to that obtained when the SDE was tested. The breakdown of endotoxin by detergents such as sodium desoxycholate into smaller sub-units was described by Ribi, Anaker, Brown, Haskins, Malmgren, Milner and Rudbach (1966). This would allow the molecules to diffuse faster through agar, altering the position of the precipitate lines as seen in Plate 11. Immunodiffusion patterns of the LPS before and after heat treatment are shown in Plate 12. The LPS gave at least two lines of precipitate, which were so close to each other that they looked like one precipitin band. However, after the LPS had been treated with heat, two discrete precipitin lines were seen. Plate 12 also shows the precipitation patterns obtained with the HE before and after treatment with phenol. Phenol-treated HE gave two lines of precipitate, which would be expected from the polydispersity of the

Plate 9 Immunodiffusion precipitin patterns of A. equuli NCTC8644 antigens against homologous antisera raised to heated vaccine (O) or formolised vaccine (F).

Outer wells contain NCTC8644 antigens. Symbols used in the Plate are explained in Table 38 (p. 191).

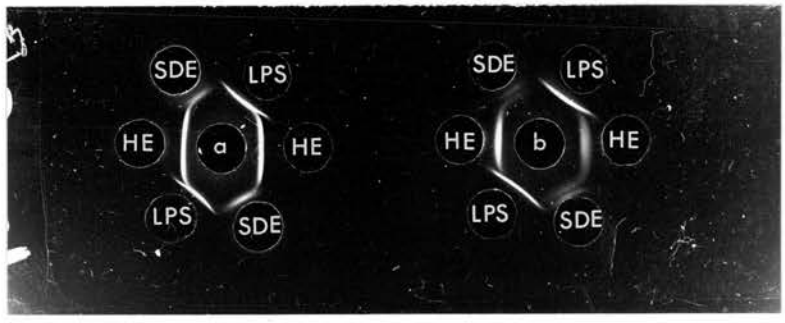

Plate 10 Immunodiffusion precipitin patterns of A. equuli antigens from strains NCTC8644 and ERS29 against their homologous antisera (a and b respectively) raised to heated vaccine.

Outer wells contain antigens.
Symbols used in the Plate are explained in Table 38 (p.191).

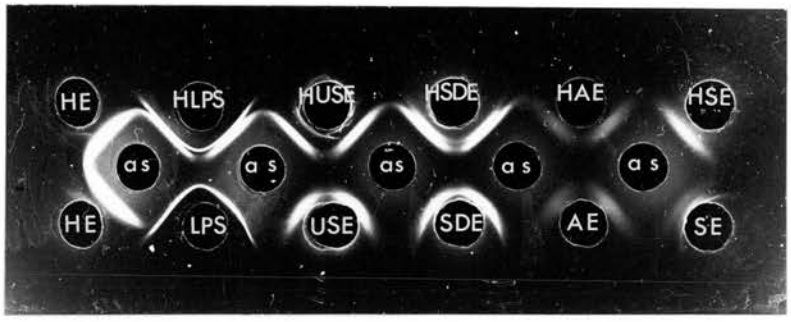

Plate 11 Immunodiffusion precipitin patterns of A. equuli NCTC8644 antigens before and after heating at 100°C for 2 hours against homologous antiserum(as) raised to heated vaccine.

Outer wells contain NCTC8644 antigens. symbols used in the Plate are explained in Table 38 (p. 191).

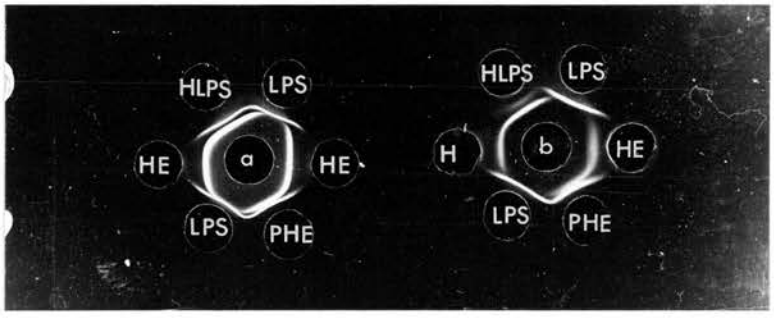

Plate 12 The effects of heat and phenol treatment on Westphal-type lipopolysaccharides and heat extracts, respectively, in immunodiffusion precipitin test.

Centre wells contain antisera raised to heated vaccines of strain NCTC8644(a) and ERS29(b). Outer wells contain homologous antigen preparations. Symbols used in the Plate are explained in Table 38 (p. 191).

phenol-water extracted preparation. The slower diffusing components from the well containing the phenol-treated HE$_A$ formed a line of identity with the HE while the faster moving band appeared to give a reaction of identity with the additional antigenic determinant on the LPS that is not found in the HE antigen. As reactions of identity of the phenol-treated HE with the LPS were obtained with antiserum prepared against the heated vaccine, it is apparent that the antigenic determinants on the HE were broken down by phenol treatment into the smaller sub-units that are present in the LPS.

(ii) Serogrouping of the strains

Immunodiffusion precipitin tests using the 28 antisera raised to the heated vaccines of A. equuli strains were carried out with heat extracts prepared from the strains to which the antisera had been raised. The results of these tests are summarised in Table 36, and the precipitin patterns of the heat extract prepared from strain ER&53 against the 28 grouping sera are presented in Plate 13. No precipitin lines were obtained when any of the antisera used in the work were tested against the preparations consisting of the ingredients contained in the culture media, nor were there any visible precipitin bands in the immunodiffusion tests when using the preinoculation sera. In general, the antisera showing high titres in the agglutination tests also gave strong reactions in the immunodiffusion tests. The

Table 36 Immunodiffusion precipitin reactions between 28 O-groups of A. equuli and their antisera

Antigen	Antiserum prepared against strains:-																												O group
	RDV1	RDV28	RDV45	RDV62	ERS3a	ERS18	ERS20	ERS22	ERS54	ERS31	ERS37	ERS40	ERS46	ERS47b	ERS53	EQ2	EQ3	EQ7	NCTC3365	NCTC8529	NCTC8644	NCTC8987	ERS12	EQ4	RDV9	RDV64	RDV44	ERS29	
RDV1	**3**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	1	1	1	1	1	1
RDV28	0	**2**	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	2	0	2	0	0	2
RDV45	0	0	**2**	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	1	0	0	0	0	1	0	0	0	0	3
RDV62	0	0	0	**2**	0	0	0	0	0	0	0	0	0	0	0	2	0	0	1	0	0	0	0	1	0	2	0	1	4
ERS3a	0	0	1	0	**2**	0	0	1	1	0	0	0	2	1	2	2	0	0	1	0	2	1	0	1	0	0	0	0	5
ERS18	0	0	0	0	1	**2**	0	0	1	0	0	1	1	0	1	2	0	0	1	0	0	1	0	1	0	0	0	0	6
ERS20	0	0	0	0	0	0	**2**	0	0	0	0	0	0	0	0	2	0	0	1	0	0	1	0	2	0	2	0	0	7
ERS22	0	0	0	0	0	0	0	**2**	0	0	0	0	0	0	0	2	0	0	1	0	0	1	0	1	0	0	0	0	8
ERS54	0	0	0	0	0	1	0	0	**2**	0	0	0	0	0	0	2	0	0	1	0	1	2	0	1	0	0	0	0	9
ERS31	0	0	0	0	0	0	0	0	0	**2**	1	0	1	0	1	2	0	0	1	0	1	2	0	1	0	0	0	0	10
ERS37	0	0	0	0	0	0	0	0	0	0	**2**	0	0	0	0	1	0	0	1	0	0	2	0	2	0	1	0	0	11
ERS40	0	0	0	0	0	0	1	0	0	0	0	**2**	0	0	0	1	0	0	1	0	0	2	0	2	0	2	0	0	12
ERS46	0	0	0	0	1	1	0	0	1	0	0	0	**2**	0	0	1	0	0	1	0	0	1	0	1	0	0	0	0	13
ERS47b	0	0	0	0	2	1	0	0	1	0	0	0	0	**2**	0	2	0	0	1	0	0	1	0	1	0	1	0	0	14
ERS53	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**>1**	2	0	0	1	0	0	1	0	1	0	0	0	0	15
EQ2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	**3**	0	0	1	0	0	2	0	1	0	1	0	0	16
EQ3	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	2	**2**	0	1	0	0	1	0	1	0	0	0	1	17
EQ7	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	**2**	1	0	0	1	1	1	0	2	0	1	18
NCTC3365	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	2	0	0	**2**	0	0	1	0	0	0	0	0	0	19
NCTC8529	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	2	0	0	1	**2**	0	1	0	1	0	0	0	0	20
NCTC8644	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	2	0	0	1	0	**2**	1	0	1	0	1	0	0	21
NCTC8987	0	0	0	0	1	1	0	0	1	0	0	1	1	1	0	2	0	0	1	0	0	**3**	0	2	1	2	0	1	22
ERS12	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	2	0	1	1	0	1	1	**3**	1	1	0	1	1	23
EQ4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	2	1	**3**	1	1	0	1	24
RDV9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	1	0	0	2	0	1	**2**	1	0	0	25
RDV64	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	1	1	1	**2**	0	1	26
RDV44	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	2	0	1	1	0	0	1	1	1	0	1	**3**	2	27
ERS29	0	0	1	0	0	0	1	1	0	0	0	0	1	1	1	1	1	0	1	0	1	1	2	1	0	1	2	**3**	28

Figures in the table indicate number of precipitin lines.

results obtained with the immunodiffusion tests, although considerable cross reactions were observed in this test with some antisera, were generally in agreement with those obtained by the tube agglutination tests. Twenty-eight specific heat-stable antigens were demonstrated, each of which reacted only with the homologous antiserum. When the strains which had been placed into one particular group on the basis of the agglutinin-absorption tests, were then examined against their group antiserum, their group characteristics were demonstrated by the formation of one or more precipitin line(s) unique to that group. Plate 14 shows that the seven strains of Group 14 tested against the antiserum raised to strain ERS47b (Group 14 antiserum), have each produced two precipitin lines showing a reaction of identity between these strains. Plate 15 shows reactions with organisms of Group 21. Strains NCTC8644 and NCTC9435 tested against the Group 21 antiserum (antiserum raised to the heated vaccine of strain NCTC8644), have each formed three precipitin lines, whereas strains RDV60, ERS51 and NCTC8794 have formed only two lines which, from the reaction of partial identity illustrated in Plate 15, can be seen to be identical with two of the three lines of the first two strains. It was found that the additional antigenic determinant in the strains NCTC8644 and NCTC9435 could be demonstrated by using antiserum NCTC8644 absorbed with RDV60, ERS51 or NCTC8794 and that

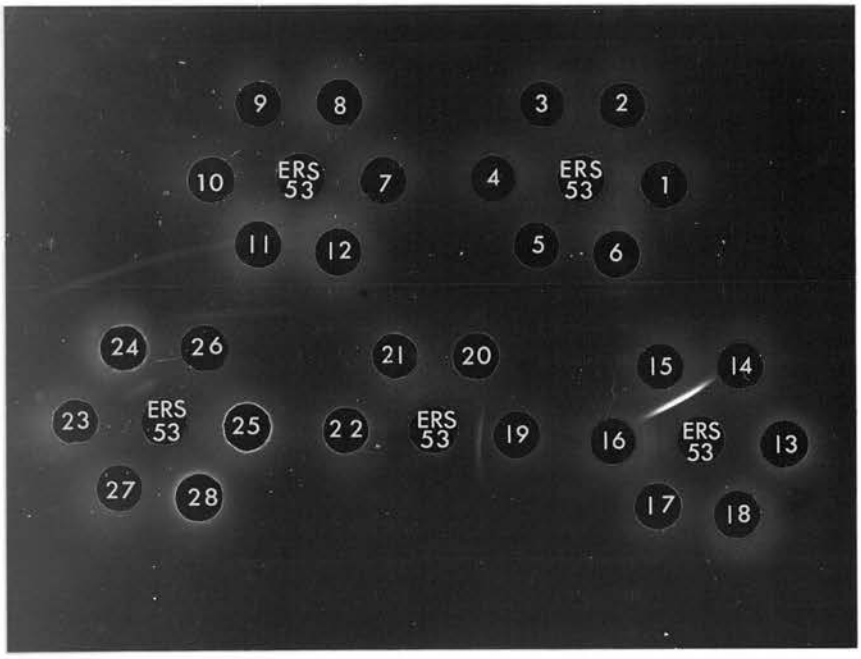

Plate 13 Immunodiffusion precipitin patterns of ERS53 against the 28 O group antisera.
Inner wells: heat extract from strain ERS53
Outer wells: 28 O grouping antisera
Notice the intense precipitin line(s) formed between O group 15 antiserum (ERS53 antiserum) and ERS53 heat extract.

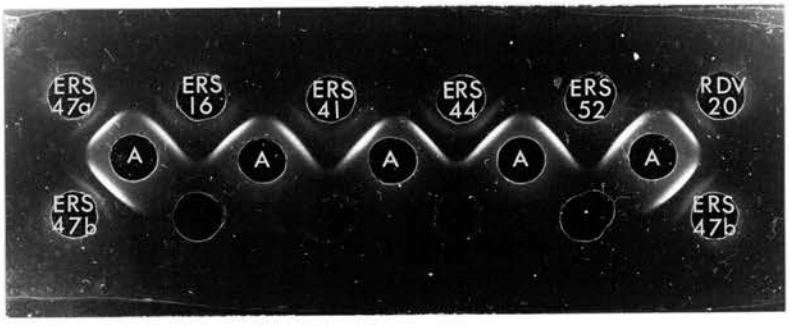

Plate 14 Immunodiffusion precipitin patterns of 7 heat extracts prepared from Group 14 strains against their group antiserum (antiserum ERS47b). The (A) wells contain antiserum and outer wells contain antigens. Notice the lines of identity between 7 strains.

Plate 15 Immunodiffusion precipitin patterns of 5 heat extracts prepared from O group 21 strains against their group antisera (serum NCTC8644). The (B) wells contain antiserum and outer wells contain antigens.

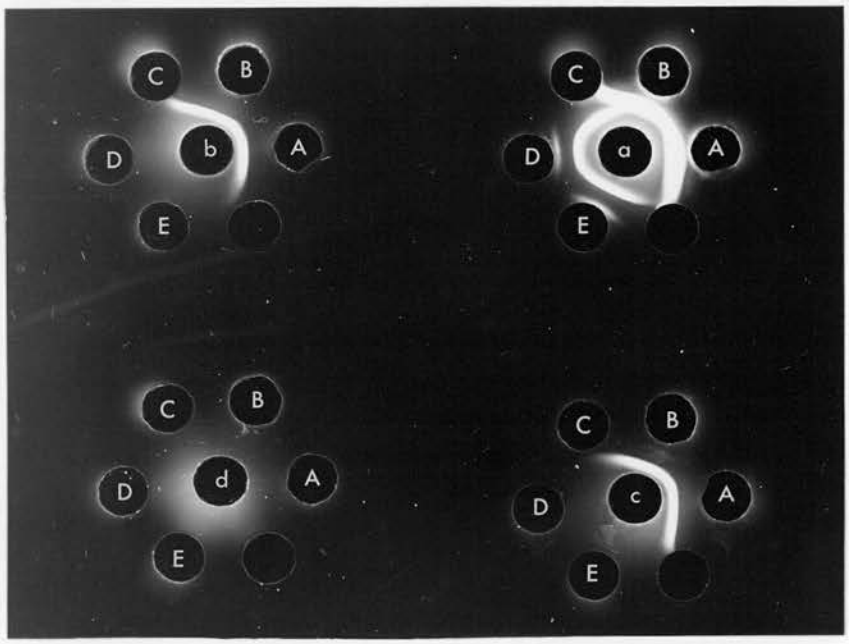

Plate 16 Immunodiffusion precipitin patterns of 5 strains of O group 21 against their group antiserum (serum NCTC8644) before and after absorption with NCTC8794, RDV6O and NCTC9435.
Inner wells: (a) NCTC8644 antiserum, (b) NCTC 8644 antiserum absorbed with NCTC8794,
(c) NCTC8644 antiserum absorbed with RDV6O,
(d) NCTC8644 antiserum absorbed with NCTC9435.
Outer wells: heat extracts of (A) NCTC8644,
(B) NCTC9435, (C) NCTC8794, (D) RDV6O, (E) ERS51

the antibody fractions reacting with the latter three strains could be absorbed by any one of the five strains arranged into O group 21. Such results are illustrated on Plate 16.

Minor antigenic differences existing between strains of O group 28 (see Table 22, p.145) could also be demonstrated in the immunodiffusion precipitin tests (Plates 17a and 17b). The five porcine haemolytic strains which fell into O group 28 in the agglutination tests produced a reaction of identity when tested against the Group 28 antiserum (antiserum raised to ERS29 heated vaccine). Reactions of identity between three strains (ERS8, ERS15 and ERS29) of O group 28 when tested against the Group 28 antiserum are illustrated on Plate 18 where the strains of O groups 23 (ERS7 and ERS10) and 27 (ERS28 and ERS38), which showed considerable cross reactions in the agglutination tests, have been tested alongside for comparison. The precipitin lines produced by the three strains of Group 28 are so marked that one can hardly confuse the line(s) unique to the Group with those produced by the strains of Groups 23 and 27, both of which appeared to share minor antigenic determinants with Group 28.

An interesting result found was that at least one antigenic determinant in the thermostable antigen complexes appeared to be shared by all 28 strains of A. equuli to which antisera had been prepared. The

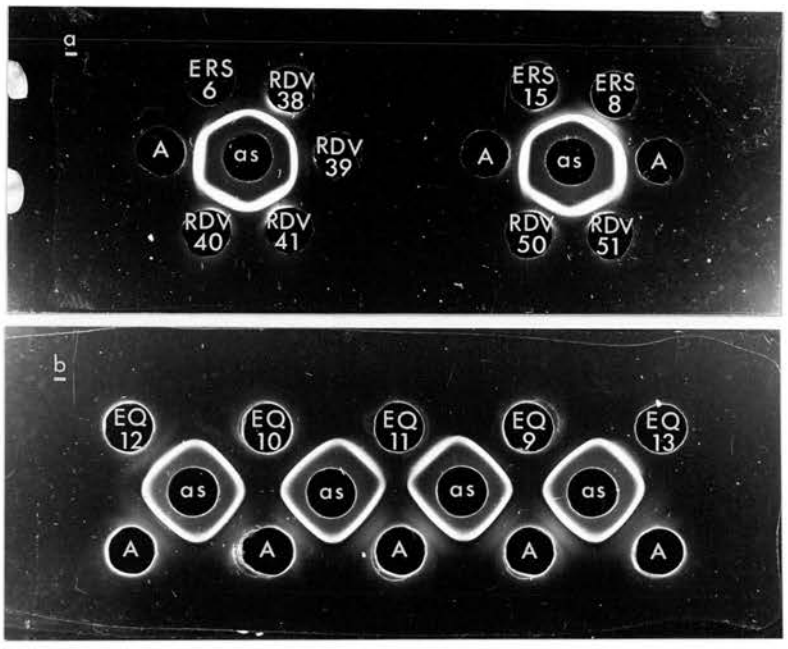

Plate 17 a: Immunodiffusion precipitin patterns of 10 strains of O group 28 against their group antiserum (serum ERS29).
b: Immunodiffusion precipitin patterns of 5 haemolytic porcine strains against O group 28 antiserum.
The (as) wells contain antiserum ERS29.
The (A) wells contain antigen ERS29.

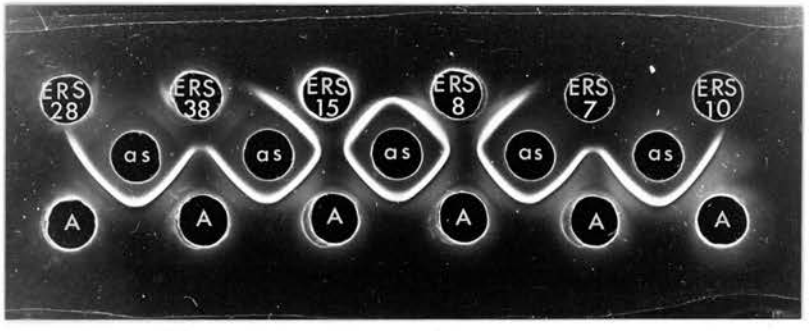

Plate 18 Immunodiffusion precipitin patterns of O groups 23, 27 and 28 against antiserum ERS29 (Group 28 antiserum).
ERS7 & ERS10 (Group 23), ERS28 & ERS38 (Group 27), ERS8, ERS15 & ERS28 (Group 28).
The (as) wells contain antiserum ERS29.
The (A) wells contain antigen ERS29.

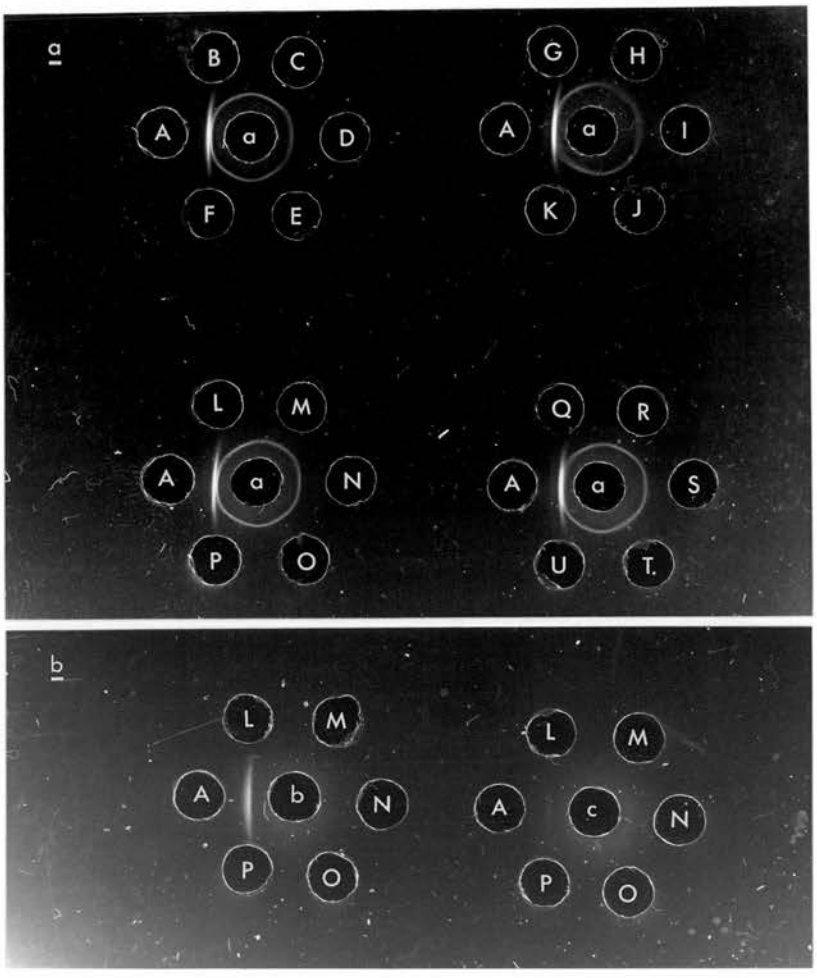

Plate 19 a: Immunodiffusion precipitin patterns of 20 different O groups of A. equuli against antiserum NCTC3365(a).

b: Antiserum NCTC3365(b) absorbed with the heat extract of strain ERS53 has retained specific antibody fraction(s) to homologous strain while no reaction has been observed when the antiserum (c) absorbed with homologous antigen. Antigens in outer wells: A = NCTC3365, B = RDV1, C = RDV28, D = RDV45, E = RDV62, F = ERS20, G = ERS22, H = ERS37, I = ERS40, J = ERS46, K = ERS47b, L = ERS53, M = EQ2, N = EQ7, O = NCTC8529, P = NCTC8644, Q = RDV9, R = RDV44, S = RDV64, T = ERS12 and U = ERS29.

common heat-stable antigenic determinant was demonstrable by the appearance of a precipitin line as a reaction of identity when all these strains were tested against the antiserum raised to the heated vaccine prepared from strain NCTC3365 (Plate 19a). Similar results were also obtained with the antisera prepared against the heated vaccine of strains EQ2, EQ4 and NCTC8987, but the common line was not observed when the other 24 antisera (see Table 36) were tested. The antibody fraction(s) reacting with the common antigenic determinant(s) on each strain could be exhausted by any one of the 28 strains to which antiserum had been raised. Such a result obtained when the antiserum NCTC3365 absorbed with the heat extract from strain ERS53 was tested, is illustrated on Plate 19b .

Immunodiffusion precipitin tests using the heat extracts of all the 138 strains of A. equuli included in this work were carried out with 10 antisera (RDV1, RDV28, ERS12, ERS22, ERS29, ERS37, ERS53, NCTC3365, NCTC8529 and NCTC8644), and the results are tabulated in Appendix D. From these results it can be seen that at least one component in the heat stable antigen complexes is shared by all strains of A. equuli included in this work.

(iii) The relationship of the immunodiffusion precipitin test to other serological tests

To determine the relationship between the various

Table 37

The effect of absorption with A. equuli heated cells or heat extract on the activity of homologous antiserum in serological tests

Antiserum to strain	Absorbing agent	Activity in serological tests*		
		TAT	PHA	IDT**
NCTC 8529	0.01M PBS	640	10240	2
	NCTC8529 heated cells	N	N	0
	NCTC8529 heat extract	160	N	0
ERS12	0.01M PBS	1280	20480	>2
	ERS12 heated cells	N	N	0
	ERS12 heat extract	320	N	0

* TAT = tube agglutination test
PHA = passive haemagglutination test
IDT = immunodiffusion precipitin test
N = no agglutination at a dilution of 1 in 20

** no. of lines with heat extract from homologous strain in the immunodiffusion precipitin test

> indicates the overlapping of precipitin lines

serological tests applied in the present study, the serological activity of two antisera to A. equuli was tested before and after absorption with the heated cells and the heat extracts prepared from the homologous strains. The results of these tests are summarised in Table 37. The absorption with the heated cells from the homologous strain appeared to remove completely antibodies active in the serum agglutination test, the passive HA test and the immunodiffusion precipitin test. Absorption with the heat extract of the homologous strain produced results essentially similar to those obtained with the heated cells. However, in the case of the agglutination test, low levels of antibody remained after double absorption with the heat extract, although repeated absorption exhausted the serum agglutinin completely.

(e) Immunoelectrophoresis

Using rabbit antisera raised to the formolised vaccines and to the heated vaccines of strains NCTC8644 and ERS29, three antigenic extracts (ultrasonic extract, Westphal-type lipopolysaccharide and heat extract) of the strains were analysed by immunoelectrophoresis (IEP). The IEP patterns obtained with strain NCTC8644 are shown in Plate 20. The antiserum produced against the formolised whole cells appeared to form more discernible lines than did the antiserum against the heated vaccine.

Two notable features are the absence of precipitin bands on the anode side of the plate with LPS or HE and the reduced number of precipitin bands formed with HE as compared with USE. However, all three antigens appear to have the same fast-migrating antigenic determinant(s) which formed precipitin band(s) on the cathode side of the origin.

When the USE of strain ERS12 was tested against its homologous antiserum raised to formolised whole cells 8 precipitin bands were formed (Plate 21). IEP patterns showing the effect of heating the USE of strains NCTC8644 and ERS12 are illustrated in Plate 22. The antigenic determinants of ERS12 migrating towards the positive pole appeared to be eliminated by heat treatment, indicating that those migrating towards the cathode are heat stable. With the USE of strain NCTC8644 slow-migrating antigenic determinants towards either positive or negative pole were sharply reduced after heat treatment. However, there were no changes in either the shape or position of the precipitin bands formed with heat-stable antigenic determinants.

To compare the results obtained with the immunodiffusion precipitin tests with those of IEP, the two techniques were used with the heat extracts prepared from 4 strains of O group 17 and 5 strains of O group 20 using their group antisera prepared against the heated cells of strains NCTC8529 (Group 20) and EQ3 (Group 17).

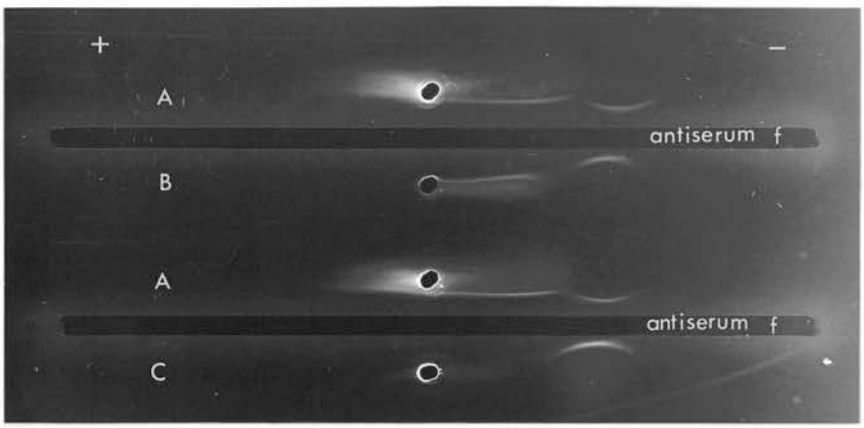

Plate 20 Immunoelectrophoretic patterns of the soluble antigens prepared from NCTC8644 against the homologous antiserum (f) raised to formolised vaccine. A = ultrasonic extract, B = Westphal-type lipopolysaccharide, C = heat extract

Plate 21 Immunoelectrophoretic precipitin patterns of the ultrasonic extracts from strain ERS12(A) and NCTC8644(B) before and after heating at 100°C for 2 hours against their homologous antiserum. USE = ultrasonic extract, HUSE = heated USE, a = ERS12 antiserum raised to formolised vaccine, b = NCTC8644 antiserum raised to formolised vaccine.

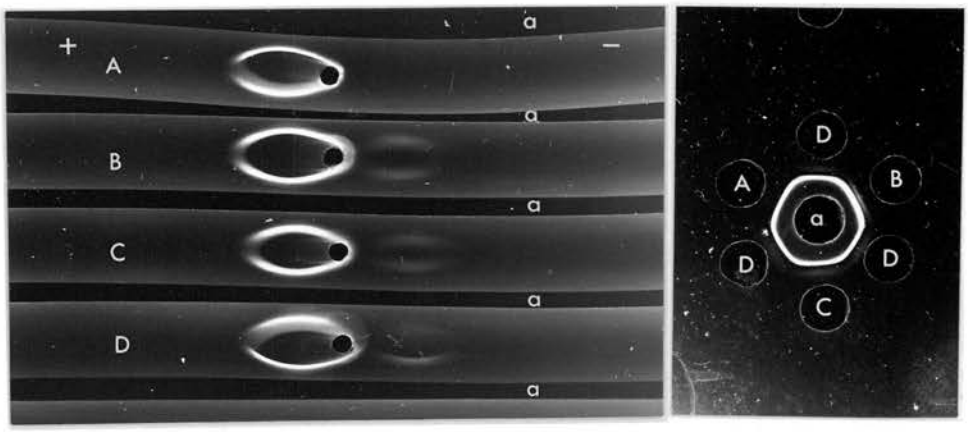

Plate 22 Immunoelectrophoretic and immunodiffusion precipitin patterns of the heat extracts prepared from 4 strains of O group 17.
A = ERS26, B = EQ1, C = EQ6, D = EQ3
a = antiserum EQ3 (O group 17 antiserum)

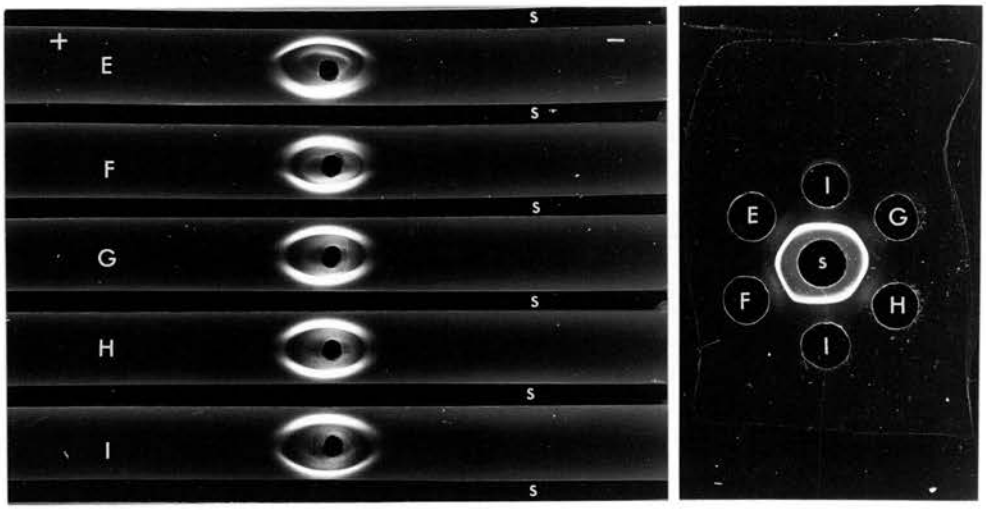

Plate 23 Immunoelectrophoretic and immunodiffusion precipitin patterns of the heat extracts prepared from 5 strains of O group 20.
E = RDV34, F = ERS23, G = ERS33, H = ERS34, I = NCTC8529, s = antiserum NCTC8529 (O group 20 antiserum)

The results obtained are presented in Plates 22 and 23. All 4 strains of O group 17 produced more than 2 bands on the anode side of the origin, and these bands produced with each of four strains appeared to be identical to one another. With the three porcine strains (EQ1, EQ3 and EQ6), one band on the cathode side of the origin was visible while no such reactions were obtained with ERS26 which was isolated from the nostrils of an apparently healthy horse. The three strains which appeared to be antigenically identical were involved in pathological processes in pigs, two having been isolated from lesions of endocarditis (one in Kenya in 1962 and the other in the U.K. in 1968) and the remaining strain having been recovered from the joint of a pig. Similar results were obtained with the immunodiffusion precipitin test where strain ERS26 produced one strong band which shows a reaction of identity with the inner band produced by three porcine strains. The additional outer band produced by these three porcine strains can be seen in Plate 22.

Five strains (NCTC8529, RDV34, ERS23, ERS33 and ERS34) of that O group 20, one of which was recovered from a foal with septicaemia and the remaining 4 strains were isolated from the upper respiratory tract of normal horses, appeared to be antigenically identical as shown in Plate 23. These findings would indicate that the results obtained with the immunodiffusion precipitin

test and the immunoelectrophoresis are in accordance with those of the agglutinin-absorption tests (see Table 22, p. 145).

Table 38

Symbols used in some plates in Part II

Symbol	Meaning
AE	Alcohol extract
HE	Heat extract
LPS	Westphal-type lipopolysaccharide
SDE	Sodium desoxycholate extract
SE	2.5% saline extract
USE	Ultrasonic extract
HAE	AE heated at 100°C for 2 hours
HLPS	LPS heated at 100°C for 2 hours
HSDE	SDE heated at 100°C for 2 hours
HSE	SE heated at 100°C for 2 hours
HUSE	USE heated at 100°C for 2 hours
PHE	HE treated with phenol

4. DISCUSSION

(a) Agglutination and agglutinin-absorption tests

Serological studies on A. equuli have been undertaken since the beginning of the 1920's primarily to provide a basis for a serological diagnosis and for serum therapy against the disease (Lütje, 1922a: Miessner and Berge, 1922; Goerttler, 1925; Edwards, 1931, 1932; Haupt, 1934). However, information on the serology of the organism is still scarce in the available literature. Moreover, previous workers who have studied the serological behaviour of A. equuli have employed a variety of antigens and antisera prepared in a number of ways, so that their results are not always strictly comparable. A number of antigen preparations such as living organisms (Miessner and Berge, 1922; Edwards, 1931; Haupt, 1934; Hirato, 1939; Mráz, 1968), heat killed bacterial suspensions (Phillips, 1966) and phenol- or formalin-killed organisms (Kowatch, 1925; Vallée et al., 1963; Wetmore et al., 1963) have been used, not only for the preparation of specific antisera, but also for the demonstration of antigen-antibody union. None of the previous workers has made a conclusive comparison of different types of antigen or antisera.

Edwards (1931), Haupt (1934) and Hirato (1939) found that their organisms were extremely heterogeneous in their serological characteristics. Merchant and

Packer (1967) stated that the species is composed of a number of heterogeneous strains which give positive agglutination and precipitation reactions only against their homologous antisera. Likewise, Doll (1963) and Knight (1972) claimed that the immunoprophylaxis of the disease is not practicable simply because of the antigenic diversity of A. equuli. Although it has been generally accepted that there is considerable serological heterogeneity between strains, no previous workers have investigated the possible existence of antigenic types of A. equuli. Thus, the antigenic structure of the organism has still remained unsolved.

The somatic (O) antigens of A. equuli studied in the present work have shown a high degree of specificity and afforded a means of classifying the strains of this rather heterogeneous species into 28 O-groups. The classification of no less than 98.6 per cent. of the organisms into specific O groups suggests that the major heat-stable antigens (O antigens) concerned with this are quite distinct. However, the existence of minor antigens, which may be common to several groups, is indicated by the occurrence of cross agglutination reactions. The results obtained with agglutinin-absorption tests provide evidence that there are some antigenic differences even within the same O groups of A. equuli. However, it is too early to classify the O groups into subgroups since no conclusive investigation

has been made on each antigenic factor. As no information is available on the form and quantitative varieties of O antigen of A. equuli, further studies would be required to confirm the stability of each O antigen factor in agglutinogenicity or immunogenicity.

The commensal and pathological opportunist status of A. equuli is further supported by the fact that none of the identified multiple serogroups of the organism is consistently associated with disease in horses (Table 39). However, it would be of interest to find out, with an increased number of strains from pigs, whether certain O groups are consistently associated with pathological processes in this species of animal, since two non-haemolytic strains isolated from endocarditis in pigs and five haemolytic strains recovered from pigs with septicaemia were of only two O groups, 17 and 28, respectively.

The results obtained with living and formolised antigens of NCTC8644 and ERS12 have shown that these antigens differ serologically from those treated with heat at 100°C for 2 hours, whilst no differences in agglutination pattern were obtained with the antigens prepared from strains NCTC3365 and NCTC8529. The difference obtained with strains NCTC8644 and ERS12 points to the existence of an envelope antigen (K antigen) which appears to be relatively non-specific, since antiserum ERS12 prepared against formolised cells reacted

Table 39

Correlation between O antigenic groups of A. equuli and their clinical condition of origin

Somatic (O) groups	Strains from pathological material		Strains from healthy animals	
	Horse	Pig	Horse	Pig
1	2	0	4	0
2	0	0	5	0
3	0	0	3	0
4	0	0	3	0
5	2	0	0	0
6	0	0	2	0
7	1	0	0	0
8	0	0	4	0
9	1	0	0	0
10	0	0	2	0
11	0	0	4	0
12	0	0	3	0
13	1	0	7	0
14	2	0	5	0
15	1	2	4	0
16	1	0	3	0
17	0	3	1	0
18	2	0	2	0
19	1	0	0	0
20	1	0	4	0
21	3	0	2	0
22	1	0	1	0
23	0	0	12	0
24	0	0	1	0
25	0	0	2	0
26	0	0	3	0
27	3	0	7	0
28	1	5	19	0
Ungroupable	0	0	2	0
Total	23	10	105	0

Figures in the table indicate the number of strains.

to living and formolised antigens of unrelated O groups. Vallée et al. (1963) have observed the existence of heat-labile antigenic fractions considered to be relatively common in their strains of A. equuli. Moreover, the possible existence of envelope (K) antigens that block somatic (O) agglutination was suggested by the agglutination patterns obtained with the homologous system of strain NCTC8644 in the slide agglutination test (see Table 19, p. 139). Phillips (1966, 1967) stated after investigating the antigenic structure of 225 strains of A. lignieresi, that the heat-labile antigens associated with extracellular slime are common to organisms of different antigenic types and they may bring about inagglutinability of living organisms tested with antisera to the heat-stable vaccines. The existence of extracellular slime produced by A. equuli had been demonstrated by microscopical methods (Phillips, 1966). Cowan (1974) has described that A. equuli is an organism that produces a copious slimy covering, but this is not capsular material. The demonstration of K antigens of A. equuli were merely of a preliminary nature, so further studies on this matter are needed.

(b) Passive haemagglutination test

The results of the present studies on bacterial haemagglutination provide evidence that the passive haemagglutination test is a sensitive procedure for the

detection of antibodies to A. equuli. Erythrocytes coated with the antigenic extracts obtained by heating bacterial suspensions were specifically agglutinated in the presence of homologous antiserum. Agglutination by antibody of inert, insoluble particles coated with soluble antigen is a useful and well established serological technique. Erythrocytes have been found to be extremely convenient passive carriers of antigen. Not only is it possible to coat almost any antigen on to their complex surfaces, but the coated cells are amongst the most sensitive indicators of antibody available. The many applications of this technique have been listed by Neter (1956), Kabat and Mayer (1961), Kwapinski (1972) and Herbert (1973).

Solutions of purified lipopolysaccharides or crude extracts of sonically disintegrated organisms or bacterial extracts obtained by heating bacterial suspensions have been used for the passive HA test (Keogh, North and Warburton, 1947, 1948; Hayes, 1951; Neter, Bertram and Arbesman, 1952; Carter, 1955; Landy, Trapani and Clark, 1955; Neter, Westphal, Lüderitz, Gorzynski and Eichenberger, 1956b; Davies, Crumpton, MacPherson and Hutchison, 1958; Biberstein, Gills and Knight, 1960). Indeed, the heat extracts and the lipopolysaccharides of A. equuli as presented in Table 25 (p. 155) appeared to be the most effective antigenic extracts for the modification of erythrocytes. It will be seen that the bacterial

suspensions exposed to low temperature provided practically inactive antigens for the modification of the erythrocytes whereas exposure to elevated temperature yielded a significantly active antigenic extract. It is interesting to note that the higher the temperature for the extraction of antigens, the more pronounced were the modifying effects. These results can be explained by two independent effects of heat, namely, release from the bacterial cells of somatic antigens into solution and activation of the soluble antigen. Moreover, the titres against the heat extract of antiserum raised to heated cells were considerably higher than those of the antiserum prepared against the formolised cells, indicating that the antiserum raised to heated cells would be expected to react better with the heat-stable antigens. Heating at 100°C for one or two hours yielded the most active of the preparations obtained by heat treatment alone. In this respect, boiling of bacterial suspensions for 2 hours seemed to be an excellent treatment for the preparation of erythrocyte-modifying antigens of A. equuli.

It is of interest to note that the limited erythrocyte-modifying capacities of the ultrasonic extract of A. equuli have been increased considerably by heat, phenol, sodium hydroxide or trypsin treatment. Similar findings with sonically treated suspensions of E. coli have been reported by Neter, Westphal, Lüderitz and Gorzynski (1956a).

They found that the sonic disintegrates (extracts), after being treated with heat or sodium hyroxide, resulted in an increase of their capacity to modify erythrocytes without losing their antigenicity. The ultrasonic extract treated with phenol according to the phenol-water extraction method of Westphal et al. (1952) proved to be one of the most active agents for the passive HA test for A. equuli. It has been reported that the bacterial lipopolysaccharides which do not adsorb, or adsorb only slightly, on to erythrocytes usually require treatment with dilute alkali or heat for maximum adsorption (Staub, 1954; Landy et al., 1955; Neter et al., 1956b; Davies et al., 1958). Moreover, the coating of erythrocytes with polysaccharides of Enterobacter aerogenes (Klebsiella aerogenes) and Enterobacter cloacae (Klebsiella cloacae) has been found to inhibit the haemagglutinating action of the viruses of influenza, mumps and Newcastle disease on such erythrocytes, but only if the polysaccharides had been heated or treated with alkali (MacPherson, Wilkinson and Swain, 1953). In contrast, the erythrocyte-modifying capacities of the lipopolysaccharides of Yersinia pestis (Pasteurella pestis) were not affected by either heat or alkali treatment (Davies et al., 1958). Similar results were found with the lipopolysaccharides of Franciscella tularensis (Pasteurella tularensis), which were not altered on heating at 100°C between pH 3.9 and

7.1 (Staub, 1954). The modifying and inhibiting activities of the polysaccharide from Franciscella tularensis were found to be stable to heat in the region of neutrality, but to be destroyed by treatment at 100°C in acid or alkaline solution (Wright and Feinberg, 1952). It has also been reported that the treatment of bacterial polysaccharides with dilute alkali can, in some instances, cause loss of the ability to be adsorbed on to erythrocytes and of the power of combination with antibody (Keogh et al., 1948).

The lipopolysaccharides of A. equuli without heat or alkali treatment appeared to be fully active with regard to the modification of erythrocytes. However, activity was increased even further when the lipopolysaccharides were treated with heat or dilute alkali. The nature of the chemical change brought about by heat or alkali treatment of lipopolysaccharides or crude antigen (heat extract or ultrasonic extract) is not known, but it has been correlated with removal of some of the lipid from the lipopolysaccharide antigens of Salmonella typhosa (Landy et al., 1955), of O-acetyl from lipopolysaccharides of Aerobacter aerogenes (Davies et al., 1958) or of sialic acid from meningococcal polysaccharide antigens (Hammond, Kingsbury and Weiss, 1968).

It was observed that pepsin treatment did not activate the erythrocyte-modifying capacities of the ultrasonic extract of A. equuli while the treatment

with trypsin seemed to be effective in conferring erythrocyte-modifying capacities to the antigens. Pepsin has its greatest activity in acid solution and acts to break down intact protein to simpler molecules. It is well known that pepsin breaks down only about one tenth of the total -CO-NH-linkages. On the contrary, trypsin acts under alkaline conditions and its proteolytic action although not extending beyond peptides does go somewhat further than that of pepsin. No attempts were made to determine the chemical changes produced by treating the ultrasonic extracts of A. equuli with pepsin or trypsin. It is possible, however, that the requirement of the enzymes for an optimum pH level for their maximum activity might have affected the erythrocyte-modifying activities of the ultrasonic extract. It has been found that heating the antigen in phosphate buffer at pH 7.3 yielded a substance which was more active with regard to the modification of erythrocytes than one prepared by heating in the same buffer at pH 4.9 (Neter et al., 1956b). Chun and Park (1956) have also reported that Shigella flexneri heat extracts obtained from bacterial suspensions treated with acid failed to modify erythrocytes, while those from the suspensions treated with alkali were proved to be an excellent erythrocyte-modifying agent. However, Hammond et al. (1968) found that digesting meningococcal polysaccharide antigens with mild alkali, pronase or neuraminidase were all equally

effective in conferring erythrocyte-modifying capacity to the antigens.

The fact that a single antigen solution (heat extract) of A. equuli could be repeatedly used to coat several batches of erythrocytes without losing its erythrocyte-modifying capacity would suggest that only a small portion of antigen was involved in the process of the modification of erythrocytes. Linz, Lecocq and Manderbaum (1961) reported that only a small portion (less than one per cent.) of the available antigens (human and bovine γ-globulin and albumin) was taken up by the erythrocytes in their system. Scheibel (1956), investigating the use of diphtheria toxoid as an erythrocyte-modifying agent in the passive HA test, observed that less than 5 - 10 per cent. of the toxoid was adsorbed and that a high surplus of antigen appeared to be necessary to obtain optimal modification. Davies et al. (1958) have found that, up to a certain level, there is a direct relationship between the amount of Yersinia pestis lipopolysaccharide to which the erythrocytes are exposed and their resultant sensitivity to antibody. When this point is reached no further increase in the haemagglutination titre is obtained as more antigen is added. The range of antigen concentrations which will satisfactorily bring about modification of erythrocytes may be quite large. A series of erythrocytes exposed to Yersinia pestis lipopolysaccharides in solutions containing from 6 to 50 µg./ml. of antigen have been found

to be equally sensitive to antibody (Davies et al., 1958). Landy (1954), using purified polysaccharide from Serratia marcescens in varying concentrations, observed that similar amounts of antigen were always absorbed, and that even an excess of as much as 250 times did not block or saturate the receptors for the subsequent adsorption of 5 additional antigens. Moreover, maximum serum titres for each antigen were obtained with erythrocytes treated with excess as well as with the predetermined adequate quantity of Serratia marcescens polysaccharide. On the other hand, an excess of antigen has been reported to cause non-specific haemagglutination (Heller, Jacobson, Kolodny and Kammerer, 1954). The soluble antigens of A. equuli used in the present work appeared to be in the range of the optimum concentration for the modification of erythrocytes since there was no sharp fall in titre when successive batches of erythrocytes were exposed to a single antigenic solution, and non-specific agglutination considered to be caused by an excess of antigen was not encountered.

Heat extracts from A. equuli were used for the passive HA test mainly because no obvious advantage of sensitivity or reproducibility appeared to be offered by purified lipopolysaccharide preparations and partly because such antigens can be more easily and economically produced. In the foregoing study, evidence has been

presented that the passive HA test is more sensitive than the conventional bacterial agglutination test for the serological typing of strains of A. equuli. The passive HA test as described should be a useful tool in the study of the antigenic structure of A. equuli. Its advantage over other serological procedures is its specificity and sensitivity. Obviously, the passive HA test developed for the study of the antigenic structure of A. equuli does not lend itself to the study of heat-labile antigens, since the latter are destroyed by heat treatment. Further studies are needed for the demonstration of heat-labile antigens and antibodies of A. equuli.

It has been shown previously that erythrocytes are capable of adsorbing several antigens, either simultaneously or consecutively (Hayes, 1951; Neter, Bertram, Zak, Murdok and Arbesman, 1952; Warner, Fales and Teresa, 1974). Such erythrocytes thus have become a useful tool as a polyvalent antigen for the detection of homologous antibodies. The passive HA test using erythrocytes treated with several antigens (polyvalent haemagglutination test) has been applied to the study of the antibody response of patients with infections due to enteropathogenic E. coli, salmonellae and shigellae (Neter et al., 1956a; Neter, Goryzynski, Gino, Westphal and Lüderitz, 1956c). The fact that multiple modification of erythrocytes with as many as six antigenic

extracts from different O groups of A. equuli appeared not to interfere with the specific haemagglutination, is of interest. Although the extreme antigenic diversity of A. equuli has been considered to be too great to allow for the use of a diagnostic serological test, the potential usefulness of the polyvalent haemagglutination test for the serological diagnosis of the disease would overcome the difficulties incurred by the serological heterogeneity of the organism. In this respect, further studies on the diagnostic potentialities of this method are needed.

(c) Immunodiffusion precipitin test and immunoelectrophoresis

The serological properties of the soluble antigens, i.e. heat extract (HE), sodium desoxycholate extract (SDE), Westphal-type lipopolysaccharide (LPS), ultrasonic extract (UE), saline extract (SE) and alcohol extract (AE), were compared in order to select the best antigenic extract for the immunodiffusion precipitin test. Under the present experimental conditions, the HE and LPS appeared to be the best antigens for the test. The HE seemed to be closely related but not identical to the LPS, indicating that its serological specificity is associated with an endotoxin (O antigen complex). Immunodiffusion precipitin reactions of a partial identity were obtained with the HE and LPS. The

antigen, presumably LPS, obtained from the HE treated with phenol has been found to form lines of identity with the LPS from the cells. This result could be explained by assuming that the HE had been broken down by phenol into smaller sub-units. The breakdown of endotoxin by detergents like sodium lauryl sulphate and sodium desoxycholate into smaller sub-units have been reported (Oroszlan and Mora, 1963; Ribi et al., 1966). It is of interest that the precipitin pattern obtained with the LPS and the SDE appeared to be identical (see Plates 9 and 10, p. 173). Revers and Heddleston (1974) reported that whole cell preparations and free endotoxins of Pasteurella multocida lost their immunogenicity on treatment with phenol although Revers, Heddleston and Rhodes (1967) had found them to be unaffected by heat. Revers and Heddleston considered it possible that phenol treatment of an antigenic complex composed of lipid, protein and polysaccharide results in the dissociation of the components, so that the lipopolysaccharides remaining in the aqueous phase are no longer immunogenic for mice, even though precipitins could be detected in rabbits after injection of the LPS.

Heat extracts and sodium desoxycholate extracts of A. equuli have been used in precipitin and immunodiffusion precipitin tests for the identification of the organism (Edwards, 1931; Hirato, 1939; Mráz, 1968; Ross et al.,

1972 and Vallée et al., 1974). Edwards (1931, 1932), using an autoclaved filtrate of A. equuli in the precipitin test, concluded that the results of the precipitin test were clear-cut and confirmed those obtained with the agglutination and agglutinin-absorption test. Similar results with a precipitin test have been reached by Hirato (1939) who used antigens extracted by boiling bacterial cells for 10 minutes.

The immunodiffusion tests using the heat extracts of the 28 O-groups of A. equuli to which antiserum had been raised have confirmed beyond doubt the existence of serologically distinct groups in relation to the heat-stable antigens (O antigens). The results obtained with these tests are generally agreed with those obtained with the agglutination, agglutinin-absorption and passive haemagglutination tests. The immunodiffusion precipitin test appeared to be the test of choice for the serogrouping of isolates of A. equuli because of its specificity and its simplicity and rapidity in performance. Apart from offering a useful means of classifying the strains of this rather heterogeneous species into the 28 O-groups, the immunodiffusion precipitin test certainly has a great advantage over the other serological methods in showing a clear proof of an antigenic relationship between O groups. All the strains in the present work have shown themselves to share at least one common heat-stable antigen which could be demonstrated by means of the immunodiffusion precipitin test. The

fact that there was not reciprocity of the cross reaction in every case is of interest since this would indicate the presence of common hapten(s) rather than complete antigen(s) in the common somatic component(s) of the organism. Edwards (1931) showed that, in spite of the fact that A. equuli formed an extremely heterogeneous serological group, there appeared to be a certain amount of group reaction within the species. Mráz (1968), using the immunodiffusion precipitin test, has also observed common heat-stable antigen(s) between 4 strains of A. equuli of which one was antigenically distinct from the other three strains. Furthermore, Vallée et al. (1974) have confirmed the existence of a common antigenic fraction between the three species of Actinobacillus, A. equuli, A. lignieresi and A. suis.

Minor antigenic differences between the strains of the same O group were demonstrated, and these results were closely related to those of the agglutinin-absorption test. Thus, the possible existence of sub-groups within certain O groups is further confirmed by the immuno-diffusion test.

Heat-stable (somatic) and heat-labile (presumably envelope) antigens of A. equuli were demonstrated by the use of immunoelectrophoresis. The advantage of separating the antigens electrophoretically before precipitation with antiserum was evident. The multiplicity of antigens associated with the ultrasonic extract of

A. equuli was often lost in the immunodiffusion precipitin test because of overlapping precipitin bands. The existence of sub-groups indicated by the agglutinin-absorption test and the immunodiffusion test is further supported by immunoelectrophoresis. There are no reports in the available literature describing immunoelectrophoresis with actinobacillus. More recently, polyacrylamide gel electrophoresis has been utilized for the identification of actinobacillus-like organisms recovered from the vaginas of sows (Ross et al., 1972) and from a horse with endocarditis (Vallée et al., 1974).

(d) Correlation between O antigenic groups and biochemical properties of A. equuli.

The O groups of A. equuli presented in this work are based upon an antigenic complex which may be analogous to that of the O groups of Salmonella organisms as indicated by the results obtained with the agglutinin-absorption test. Such a classification was found to be fairly well correlated with certain biochemical characters of the organisms. The correlation between O antigenic groups and the biochemical activities of A. equuli strains are shown in Table 40. The strains belonging to O groups 1 to 22, which comprised 60.1 per cent. of the strains studied, showed marked differences in some biochemical behaviours, viz. aesculin hydrolysis, haemolysis of sheep red cells and fermentation of

Table 40

Correlation between some biochemical and fermentative properties and O antigenic groups of A. equuli

Somatic (O) groups	Property				
	Aesculin	Haemolysis	Cellobiose	Salicin	Mannitol
1	0	15	0	0	100
2	0	0	0	0	100
3	0	0	0	0	100
4	0	0	0	0	100
5	0	0	0	0	100
6	0	0	0	0	100
7	0	0	0	0	100
8	0	0	0	0	100
9	0	0	0	0	100
10	0	0	0	0	100
11	0	0	0	0	100
12	0	0	0	0	100
13	0	0	0	0	100
14	0	0	0	0	86
15	0	0	0	0	100
16	0	0	0	0	100
17	0	0	0	0	100
18	0	0	0	0	100
19	0	0	0	0	100
20	0	0	0	0	100
21	0	0	0	0	100
22	0	0	0	0	100
23	75	75	42	67	92
24	100	100	100	100	0
25	100	100	100	100	0
26	100	100	100	100	0
27	100	100	100	100	0
28	92	100	92	92	0

Figures in the table indicate the percentage of strains giving positive reaction.

cellobiose, salicin and mannitol, when compared with those of the five O groups, 24, 25, 26, 27 and 28. The strains of remaining O group, 23, differed considerably in these biochemical characters from those of the above groups. It seems likely that there is sufficient justification for assigning the strains to one of the two distinct divisions and one possible intermediate division. The term 'biotype' is applied for the differentiation of these divisions of A. equuli strains since such divisions are fairly well distinguished by certain biochemical characteristics of the organism.

The contrasting characteristics of the biotypes of A. equuli are summarised in Table 41. All Type I strains, with the exception of one, gave a positive reaction with mannitol, whilst their reactions with salicin and cellobiose were negative until the 14th day. All strains composing this type were aesculin-negative and, with the exception of one, non-haemolytic. These strains fell into O groups 1-22 inclusive. On the other hand, all Type II strains were mannitol-negative, whilst the majority (92.9%) of the strains fermented salicin and cellobiose within 14 days and hydrolysed aesculin, and all haemolysed sheep erythrocytes. The Type II strains were made up of O groups 24-28 inclusive. As was to be expected, there were some strains which could not easily be placed into either Type I or Type II. The majority of the organisms comprising this intermediate type

Table 41

Characteristics of the biotypes of Actinobacillus equuli

Biotype	Type I	Intermediate type	Type II
No. of strains	84	12	42
O groups	1 to 22	23	24 to 28
Biochemical properties *			
Fermentation of:-			
Mannitol	99.8	91.7	0
Sorbitol	15.5	75.0	2.4
Salicin	0	66.7	92.9
Cellobiose	0	41.7	92.9
Hydrolysis of aesculin	0	75.0	92.9
Haemolysis of sheep red cells	1.2	75.0	100

* Figures in the Table for the biochemical properties indicate the percentage of strains showing positive reactions.

fermented mannitol and salicin, hydrolysed aesculin and haemolysed sheep red blood cells. On close examination of the biochemical characteristics of the strains, it was noted that most of the organisms of this group attacked sorbitol.

The differences in the biochemical and serological characteristics of Type I and Type II of A. equuli are so impressive, as presented in Table 41, that the question arose as to whether the two distinct biotypes should actually be assigned to one species only. In reviewing the literature, it is certain that the biochemical characteristics of Type II are rather similar to those of A. suis (Frederiksen, 1973; Mair et al., 1974; Cowan, 1974). In particular, five Type II strains recovered from pigs with septicaemia were so strikingly similar to A. suis in their biochemical characters that further studies have been carried out and the results of these investigations are presented in Part III.

Part III: The relationship of *Actinobacillus equuli* with *Actinobacillus suis*

1. INTRODUCTION

Actinobacillus suis, according to Magnusson (1931), was first described by Degen (1907) who isolated a Gram-negative, highly pleomorphic bacillus from pigs with purulent nephritis and who also named it Bacillus polymorphus suis. Some years later Magnusson (1931) in Sweden confirmed the organism as an important cause of specific nephritis in piglets. He further considered that the organism is closely related to Bacterium viscosum equi and Bacterium purifaciens, which nowadays are commonly known as Actinobacillus equuli and Actinobacillus lignieresi, respectively.

Sato and Sugimura (1955) investigated a number of haemolytic organisms resembling actinobacilli from pigs which had been submitted to the potency test for swine fever vaccine. Finding that all their 68 strains of haemolytic organism differed from Bacterium pyosepticum viscosum equi (Actinobacillus equuli) in their ability to ferment arabinose and salicin and inability to produce acid from mannitol, they claimed that the organism should be differentiated from Bacterium pyosepticum viscosum equi and that the name Bacterium pyosepticum (viscosum) is most suitable for the organism. Furthermore, they suggested that the organism should be included in the Pasteurella group in accordance with their etiological as well as biological characters.

Terpstra and Akkermans (1955) in Holland investigated a number of haemolytic strains from septicaemia, meningo-encephalitis, pneumonia and endocarditis in piglets. Noting the significant differences from Shigella equuli (A. equuli)in the fermentation of mannitol and in the haemolytic activity, they concluded that their organisms differ from that of joint-ill in foals and gave the new species name Shigella haemolytica.

van Dorssen and Jaartsveld (1962) reported the isolation of an haemolytic organism which has many characteristics in common with Actinobacillus equuli, but which differs from it by the haemolysis of horse blood agar and by the non-fermentation of mannitol. They remarked upon the fact that the same organism had been found by Degen (1907) and Magnusson (1931) to be closely associated with disease in pigs. Noting that the name Bacillus polymorphus had been used previously by Frankland and Frankland in 1887 for a Gram-negative saprophyte, van Dorssen and Jaartsveld (1962) named their organism Actinobacillus suis.

Zimmermann (1964) also isolated an haemolytic organism, probably the same organism examined by Magnusson (1931), Terpstra and Akkermans (1955), Sato and Sugimura (1955) and van Dorssen and Jaarstveld (1962), from six cases of septicaemic disease in piglets. He obtained similar results with those described by van Dorssen and Jaartsveld

(1962), and proposed the name Actinobacillus suis for his strains although he was not acquainted with the work done by van Dorssen and Jaartsveld (1962).

Bouley (1966) has isolated A. suis from a sow with acute nephritis. He also stressed the haemolytic character of the organism in comparison with A. lignieresi and A. equuli.

Recent reports (Cutlip et al., 1972; Ross et al., 1972; Bell, 1973; Windsor, 1973; Mair et al., 1974; Report, 1975) have again directed attention to actinobacillosis in piglets caused by an haemolytic organism. However, it is apparent from a review of the available literature that the taxonomic status of the haemolytic actinobacilli is still doubtful (Wetmore et al., 1963; Mráz, 1968; Cutlip et al., 1972; Ross et al., 1972; Windsor, 1973). Moreover, haemolytic organisms resembling A. suis have also been isolated from horses with pathological processes (Wetmore et al., 1963; Mráz, 1968; Carter et al., 1971; Larsen, 1974; Report, 1975).

Wetmore et al. (1963) have reported the isolation of haemolytic actinobacilli from irradiated pigs and from the foetal membrane of a foal, and they were found to be very similar in their biochemical and cultural properties. Furthermore, they considered that the biochemical and serological differences between A. lignieresi and A. equuli were insufficient to warrant the separation of these two species and they identified their

haemolytic isolates recovered from both pigs and a foal as A. lignieresi. Mráz (1968), on the basis of a comparative study of the biochemical and serological properties between his strains from horses and the porcine haemolytic strains recovered by van Dorssen and Jaartsveld (1962) and Wetmore et al. (1963), proposed to retain A. equuli as the species name for the haemolytic actinobacilli. Finding haemolytic organisms from horses with various disease conditions, Larsen (1974) took the view that the arguments for the creation of the new species name A. suis are open to discussion, since the pig is not the only host of these microorganisms. However, Frederiksen (1971, 1973), Ross et al. (1972) and Mair et al. (1974), who made investigations into haemolytic porcine strains including those of van Dorssen and Jaartsveld (1962) and Wetmore et al. (1963), claimed that the porcine haemolytic strains differed sufficiently to warrant their retention in a separate species A. suis, which name was first used by van Dorssen and Jaartsveld (1962).

It is the purpose in this section of the present work to compare further the haemolytic strains from horses and pigs in the present series with the haemolytic strains identified as A. suis and, possibly, to relate them with the presently recognised species of actinobacillus.

2. MATERIALS AND METHODS

(a) Sources of strains

Sixteen strains of *Actinobacillus suis* and 13 strains of *Actinobacillus lignieresi* together with two strains each of *Pasteurella haemolytica* and *Pasteurella multocida* were used in this section of the work. Of the 16 *A. suis* strains, thirteen (1688/72, 27585/72, 927/73, 928/73, 15296/73, 18808/73, 18809/73, 32452/73, 36659/73, 37558/73, 4015/74, NCTC10841 and NCTC10843) were provided by Dr. N.S. Mair, Public Health Laboratory, Groby Road Hospital, Leicester, England and the other 3 strains (P247 = Akkerman 53579, P380 = Wetmore 1384 and P393 = Zimmermann 2436) were supplied by Dr. W. Frederiksen, Statens Seruminstitut, Aalborg, Denmark. The thirteen strains of *A. lignieresi* and two strains each of *P. haemolytica* and *P. multocida* were chosen from the Culture Collection of the Royal (Dick) School of Veterinary Studies, Edinburgh. *A. lignieresi* strains were A2, A3, A5, A6, A7, A13, A17, A20, A43, A49, A53 and A100 and two strains each of *P. haemolytica* and *P. multocida* were M878/75, M1012/75, M825/75 and M873/75, respectively.

(b) Media and reagents

Unless otherwise stated, media and reagents used in this part of work were the same as those used in Parts I and II.

(c) Morphological, cultural and biochemical properties

Each strain of A. suis and A. lignieresi in the present work was subjected to the same tests described in Part I and these tests were carried out by the same methods as in Part I.

(d) Preparation of antigens

Antigens for the preparation of antisera and for the serological tests were also prepared as in Part II.

(e) Antiserum production

Antisera against the heated vaccines of 2 strains each of A. suis (NCTC10841 and P393) and A. lignieresi (A5 and A7) were raised in rabbits using the method employed for antisera against A. equuli (p.122). Antisera against A. equuli used in this part of work were the same antisera used in Part II.

(f) Serological procedures

Serological tests used in the work were those described in Part II.

3. RESULTS

(a) Morphological and cultural characters of A. suis

All sixteen strains of A. suis examined in the work were non-motile, Gram-negative bacilli. In the majority of cases coccobacillary and short bacillary forms were predominant although the morphology of the organisms appears to vary considerably with the medium on which they were grown. Often these short bacillary forms revealed a distinct bipolar appearance as seen with the majority of the strains of A. equuli. Cultures grown on Loeffler's serum slopes or on nutrient agar showed mostly short bacillary and coccobacillary forms (Plates 25 and 28), but the blood agar culture of the same strain revealed considerable lack of uniformity in morphology (Plate 24). Long rod forms and filamentous forms were not uncommonly observed in films prepared from 24 hour cultures on blood agar (Plate 26). These filamentous forms often were broken down into short bacillary forms and granules, thus giving an appearance of streptobacillary forms. Many bizarre forms were seen in most cases especially when the organism was grown on glucose or maltose agar. Swollen rods and large yeast-like bodies were the most common unusual forms seen in the smears made from glucose agar cultures (Plate 27). Faintly staining swollen rods resembling the ghost forms observed with A. equuli and A. lignieresi were also found often mixed with an

Plate 24 24-hour blood agar culture of A. suis (strain NCTC10841) showing pleomorphism of the organism. Gram. X1500.

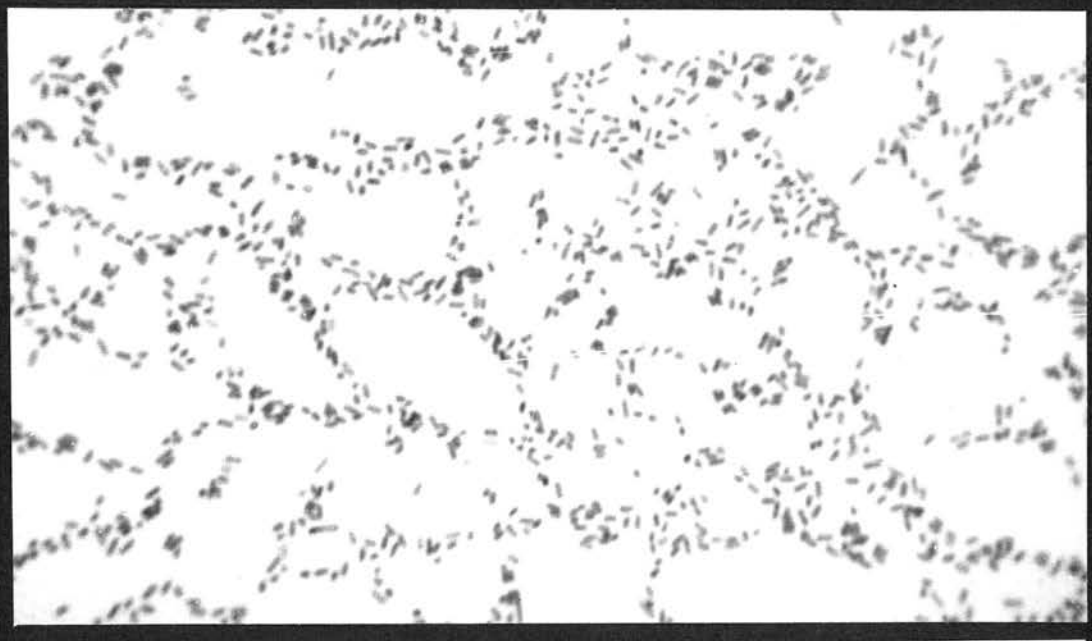

Plate 25 24-hour Loeffler's serum slope culture of A. suis (strain NCTC10841) showing short bacillary and coccobacillary forms. Gram. X1500.

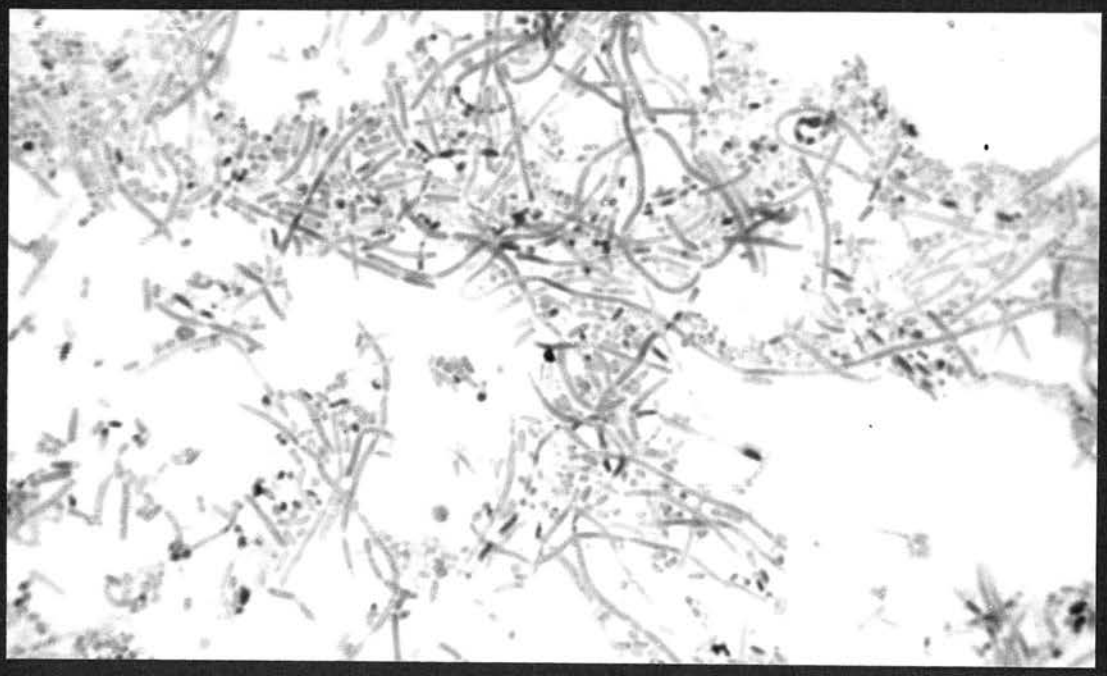

Plate 26 24-hour blood agar culture of A. suis (strain P393) showing filamentous forms. Gram. X1500.

Plate 27 24-hour glucose agar culture of A. suis (strain P393) showing yeast like bodies(y). Gram. X1500.

Plate 28 24-hour nutrient agar culture of A. suis (strain P393) showing mostly bacillary and coccobacillary forms. Gram. X1500.

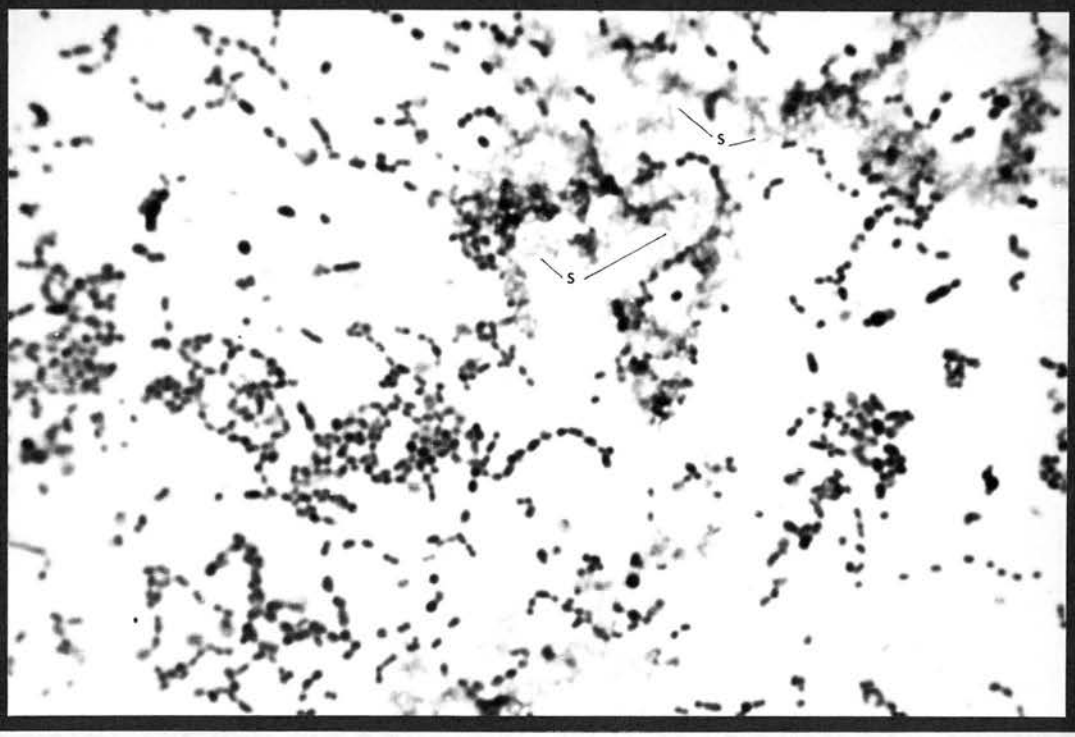

Plate 29 48-hour glucose broth culture (ropy sediment) of A. suis (strain P393) showing strepto-bacillary chains and amorphous substance (extracellular slime)(s). Gram. X1500.

amorphous substance. The organism grown in nutrient broth and glucose broth showed predominantly short bacillary forms, which were often interspersed with an amorphous substance, thus giving typical streptobacillary chains (Plate 29).

Growth occurred aerobically and anaerobically in ordinary culture media at 37°C, and it appeared to be enhanced by the addition of serum. In general, the growth on blood agar of the majority of strains of A. suis was poorer than that of A. equuli. However, the colonies on blood agar attained a size of 0.5 to 1 mm. in diameter within 24 hours and gradually increased up to 2 to 3 mm. in diameter with prolonged incubation, but the tiny pin point colonies often encountered in the initial plate from freeze dried cultures usually failed to increase their measurement with further cultivation.

The colonies on the blood agar and nutrient agar cultures were smooth, low convex, entire edged and greyish white in colour. The centre of the colony was opaque and surrounded by a clear peripheral zone. On further cultivation these colonies tended to be dome shaped mainly by the projection of transparent border zone, thus giving an appearance of fried egg or Mexican hat. The majority of strains produced viscous colonies on agar plates, and often these colonies were adherent to the medium. The sticky nature of the colonies appeared to be more pronounced with prolonged incubation (48 to 72

hours). However, the sticky nature of the colonies of A. suis was less evident than with A. equuli.

The majority of strains (87.5%) formed a ropy sediment in nutrient broth within 48 hours, while two strains, NCTC10843 and P380, failed to do so. However, the ropy nature of the broth deposit was less prominent than that of A. equuli.

The most characteristic feature of the organism grown on blood agar was a distinct zone of haemolysis around the colonies on this medium. All the strains of A. suis in the work produced a wide zone of complete haemolysis on sheep blood agar, but a zone of partial haemolysis on horse blood agar was obtained with the majority of the strains. The zone of haemolysis, in general, appeared to extend and become more marked on further incubation or on storage at room temperature.

(b) Biochemical and fermentative characters of A. suis

All the strains of A. suis in the work gave positive results in the tests for catalase, β-galactosidase, phosphatase and urease activities, nitrate reduction, hydrolysis of aesculin and the ability to grow on MacConkey's medium, while negative results were obtained in every case with tests for the ability to produce ammonia from peptone, indole production, M.R. and V.P. reactions, malonate utilisation, phenylalanine deamination, the hydrolysis of gelatin, gluconate oxidation, KCN test, citrate utilisation

and the starch-forming property from glucose. Although most biochemical characters of the organism appears to be considerably constant, some minor discrepancies between strains were observed with the remaining biochemical tests, and these are summarised in Table 42 and presented individually alongside the results obtained with 13 strains of A. lignieresi in Appendix E. A positive reaction in the test for the hydrolysis of hippurate was obtained with 15 of 16 strains, but a negative result was recorded in 15 of 16 cases in the test for the ability to produce hydrogen sulphide.

All the strains of A. suis appeared to produce a non-diffusible pigment similar to that observed with the 5 porcine haemolytic strains classified provisionally into A. equuli and examined in Parts I and II of the present series. Although pigment produced by the organism was not easily differentiated from the colour of nutrient agar or milk agar, the creamy yellow colouration of the organism was without doubt discernible if the bacterial growth was washed and suspended in saline solution. More clear evidence of the colouration of the organism was obtained by chance when the bacterial cells were ultra-sonically disintegrated.

Remarkably uniform results in the fermentative characters of the organism were obtained with every strain of A. suis included in the work, All the strains without any exception fermented arabinose, xylose, dextrose,

Table 42

Biochemical and fermentative reactions of 16 strains of A. suis

Test for	No. of strains	
	Positive	Negative
Oxidase activity	9(5)	7
Hippurate hydrolysis	15	1
H_2S production	1	15
Methylene blue redn.	8(5)	8
Starch from maltose	(3)	13
Fermentation of		
Arabinose	16(3)	0
Galactose	16(6)	0
Mannose	16(8)	0
Cellobiose	16(8)	0
Melibiose	16(2)	0
Dextrin	16(2)	0
Starch	(2)	14
Glycerol	(16)	0

Figures in parentheses indicate no. of strains showing weak positive or late positive reaction.

fructose, galactose, mannose, cellobiose, lactose, maltose, melibiose, sucrose, trehalose, raffinose and dextrin mostly with prompt reaction. Glycerol was fermented by every strain but with late reaction in the range of 3 to 6 days. Non-fermentation of rhamnose, inulin, adonitol, sorbitol, dulcitol and inositol was recorded with all the strains. In general, the fermentative activities of A. suis appeared to be greater than those of A. equuli.

(c) Cultural and biochemical characters of 13 strains of A. lignieresi.

In the course of the biochemical and serological studies of A. equuli, it was thought that the comparison of the biochemical and cultural characters of three species of Actinobacillus, viz. A. equuli, A. suis and A. lignieresi, would be necessary to produce a reliable criterion for the differentiation of these rather taxonomically disputed species of veterinary importance. For this purpose, thirteen strains of A. lignieresi were selected from the Culture Collection of the Royal (Dick) School of Veterinary Studies, Edinburgh. These strains were chosen as representing not only their host species of origin, cattle, and sheep, but also each of the antigenic types of Phillips (1966, 1967). The cultural, biochemical and fermentative characteristics of these strains were investigated by the same methods

employed for those of A. equuli and A. suis in the work, and the results obtained are presented individually, alongside the results with the 16 A. suis strains, in Appendix E.

In general, the cultural characters of A. lignieresi were similar to those of A. equuli although the sticky nature of the colonies on agar medium appeared to be less evident than that of the latter. It is of interest to note that the viscous nature of the growth seen in nutrient broth cultures of A. equuli and A. suis in the work, was hardly observed in the nutrient broth cultures of A. lignieresi in the present study.

All 13 strains of A. lignieresi gave positive reactions in the tests for catalase, β-galactosidase, phosphatase and urease activities, the production of hydrogen sulphide, nitrate reduction, methylene blue reduction and the ability to grow on MacConkey's medium. Negative results in every case were obtained with tests for the ability to produce ammonia from peptone, citrate utilisation, KCN tolerance, the production of indole, M.R. reaction, malonate utilisation, phenylalanine deamination, the hydrolysis of aesculin and gelatin and the haemolytic activities on horse and sheep blood agar. Some differences between strains were observed with V.P. reaction, oxidase activity and starch forming property of the organism, and these are given in Table 43.

Acid but no gas was produced in every case from xylose,

Table 43

Biochemical and fermentative properties of 13 strains of A. lignieresi

Test for	No. of strains	
	Positive	Negative
Oxidase activity	12(7)	1
Starch from glucose	7(5)	6
Starch from maltose	11(2)	2
V.P. reaction	1	12
Fermentation of		
Galactose	13(4)	0
Mannose	13(1)	0
Lactose	(13)	0
Maltose	13(2)	0
Raffinose	(2)	11
Dextrin	13(7)	0
Glycerol	(13)	0

Figures in parentheses indicate the number of strains showing weak positive or late positive reaction.

glucose, fructose, galactose, mannose, maltose, sucrose and mannitol, mostly with prompt reaction. All the strains attacked lactose and glycerol but only with delayed reaction. None of the strains, however, fermented arabinose, rhamnose, cellobiose, melibiose, trehalose, inulin, starch, adonitol, sorbitol, dulcitol, inositol and salicin. Discrepancies between strains were seen with the fermentation of raffinose and only 2 strains (15.4%) in the work fermented this substrate but with delayed reaction (Table 43).

(d) Serological characteristics of A. suis

The two antisera raised to the heated vaccine of strains NCTC10841 (British strain) and P393 (German strain) agglutinated all the slide antigens prepared from the 16 A. suis strains examined in the work. Antisera NCTC10841 and P393 agglutinated every strain of A. suis in the tube test to a titre comparable to that obtained with their homologous antigens and showed strong cross-absorption of agglutinins (Table 44). All the A. suis strains studied appeared to be very closely related to each other antigenically, this fact being suggested by the results obtained with the agglutinin-absorption test and immunodiffusion precipitin tests (Plate 30). However, minor antigenic differences existing between strains were observed with the immunodiffusion precipitin tests and immunoelectrophoresis. Strains 927/73, 928/73,

Table 44

Agglutination titre of two A. suis antisera before and after absorption with heated cells of ten A. suis strains

Antiserum against strain		Agglutinable and absorbing antigens prepared with strain:-									
		NCTC 10841	P393	P247	P380	928 /73	15296 /73	18808 /73	36659 /73	4015 /74	NCTC 10843
A. suis NCTC10841	a*	1280	640	640	320	320	640	320	320	640	640
	b		0	0	0	0	0	0	0	0	0
A. suis P393	a	640	640	640	320	640	640	640	640	640	1280
	b	0		20	40	0	0	0	0	0	0

* a = reciprocal titre before absorption with strain indicated

b = reciprocal titre with homologous organism after absorption with strain indicated

0 = no agglutination at a dilution of 1 in 20.

18808/73, 18809/73 and 36659/73 appeared to have one additional precipitin line compared with the other strains as shown in Plate 30. Immunoelectrophoretic analysis of 4 British strains (NCTC10841, NCTC10843, 927/73, 4015/74) and one strain each of American (P380), Dutch (P247) and German (P393) origin were carried out using the antiserum raised to NCTC10841 heated cells and the results with these tests are shown in Plate 31. Three British strains (NCTC10841, NCTC10843 and 4015/74), the Dutch strain (P247) and the German strain (P393) gave identical immunoelectrophoretic patterns while the remaining two strains, P380 (American strain) and 927/73 (British strain) appeared to form at least an additional band which was not developed by the other strains. Obviously, the additional band formed near the original well with strain P380 is clearly shown in Plate 31. Although strain 927/73 gave an intensive homologous reaction against the antiserum to NCTC10841, it can be distinguished from the other strains by the additional precipitin band formed on the cathode side of the origin.

Using 2 antisera prepared from A. suis strains NCTC10841 and P393, five haemolytic porcine strains (EQ9 to EQ13) which were grouped into O group 28 of A. equuli were compared with 2 strains of A. suis to which antisera had been raised. Results obtained with agglutination and agglutinin-absorption tests are

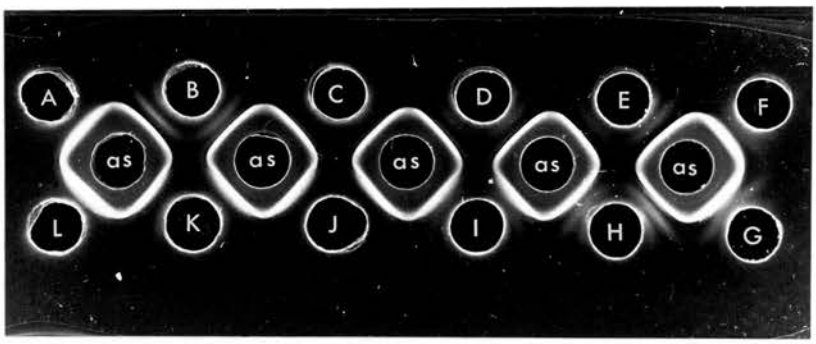

Plate 30 Immunodiffusion precipitin patterns of 12 strains of A. suis against antiserum(as) to A. equuli ERS29. A. suis antigens in outer wells: A=37558/73, B=36559/73, C=32452/73, D=18809/73 E=18808/73, F=15296/73, G=928/73, H=927/73, I=27585/73, J=1688/72A, K=NCTC10843, L=NCTC10841.

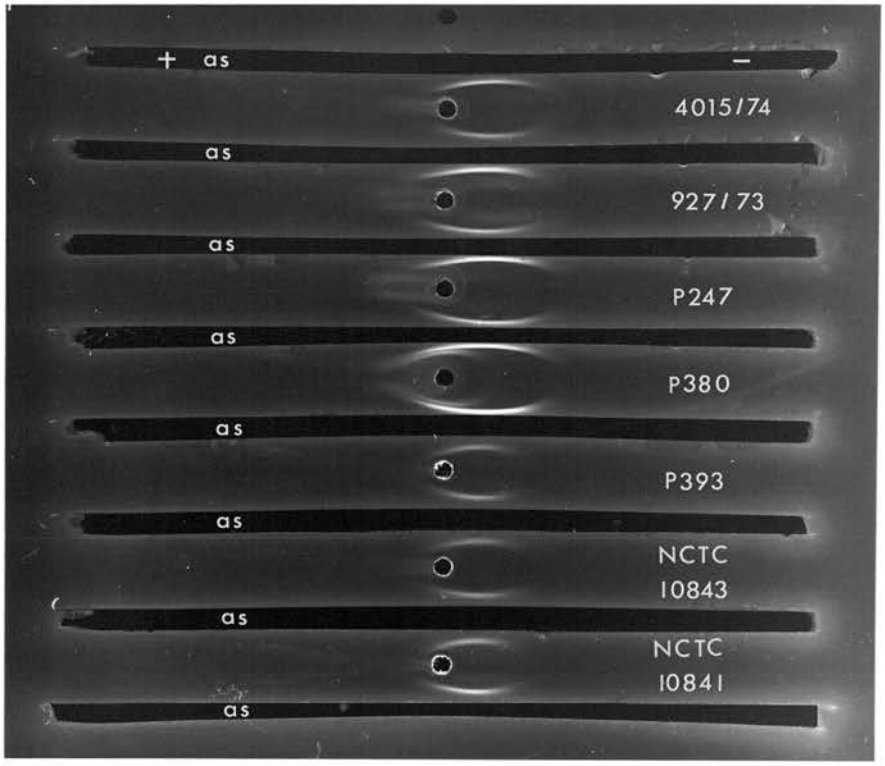

Plate 31 Immunoelectrophoretic precipitin patterns of 7 strains of A. suis against one antiserum(as) raised to A. suis NCTC10841.

presented in Table 45 and those with immunoelectrophoresis are shown in Plate 32. Each of the 5 haemolytic porcine strains of A. equuli shows a marked homologous reaction against 2 antisera raised to A. suis strains suggesting that these porcine strains are antigenically identical to those of A. suis as far as serum agglutinin is concerned (Table 45). Further evidence of the antigenic similarity between A. suis and the haemolytic porcine strains are illustrated in Plate 32 where all the strains developed very similar immunoelectrophoretic patterns against antiserum NCTC10841. In view of these results, further comparison of A. suis with two haemolytic equine strains (ERS29 and RDV55) of A. equuli Type II, which were classified together with the 5 haemolytic porcine strains into O group 28, was made to determine whether they are antigenically related to one another. The results obtained with the agglutinin-absorption(Table 46), passive haemagglutination (Table 47), passive haemagglutination-inhibition (Table 48) and immunodiffusion precipitin tests (Plate 33) appear to suggest that there are marked antigenic similarities between A. suis and O group 28 of A. equuli. That minor antigenic differences exist between these two groups of organism, however, was observed in the agglutinin-absorption and immunodiffusion tests (Table 46 and Plate 33).

The two antisera raised to strains of A. suis were tested against the heated slide antigens of the 28

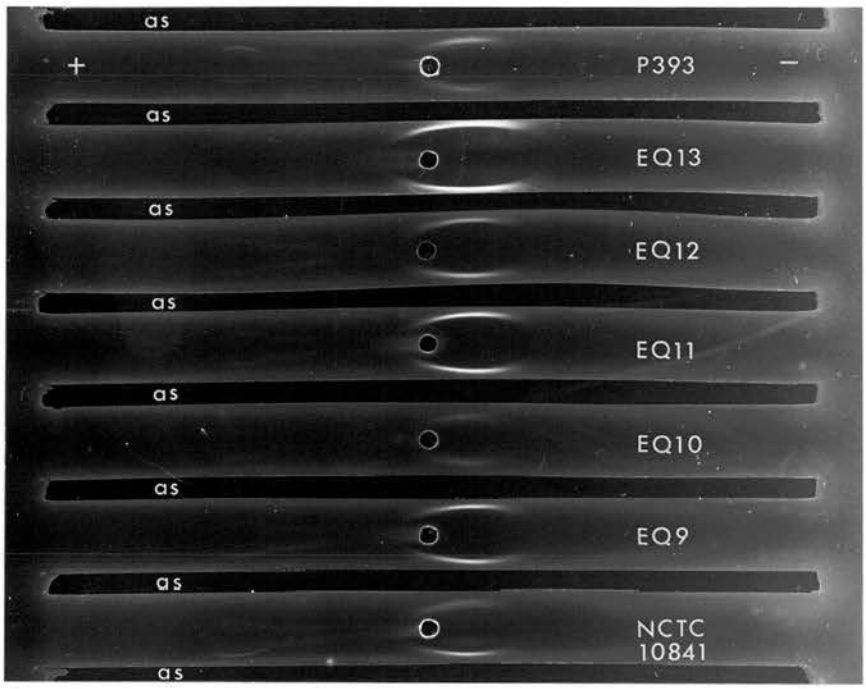

Plate 32 Comparative immunoelectrophoretic precipitin patterns of A. suis (NCTC10841 & P393) and 5 porcine haemolytic A. equuli (EQ9 to 13) against antiserum(as) to A. suis NCTC10841.

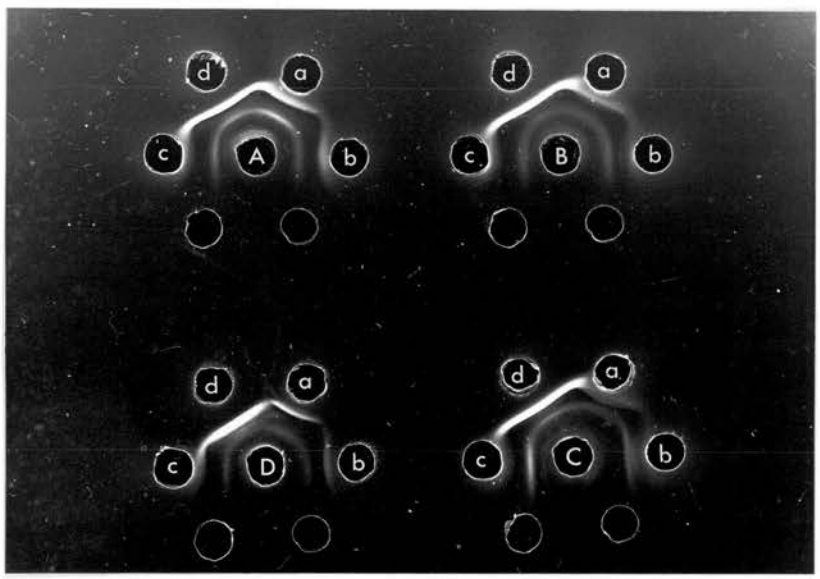

Plate 33 Immunodiffusion precipitin patterns of A. suis (NCTC10841 & P393) and equine haemolytic A. equuli (ERS29 & RDV55) against their homologous and heterologous antiserum. Antigens in centre wells: A = RDV55, B = NCTC10841, C = P393, D = ERS29 Antisera in outer wells: a = RDV55, b = NCTC10841, c = P393, d = ERS29.

Table 45

Agglutination titre of two A. suis antisera before and after absorption with heated cells of five haemolytic porcine strains of A. equuli

Antiserum against strain		Agglutinable and absorbing antigen prepared with strain:-						
		NCTC 10841	P393	EQ9	EQ10	EQ11	EQ12	EQ13
A. suis NCTC10841	a*	1280	640	640	640	640	640	640
	b		0	0	0	0	0	0
A. suis P393	a	640	640	640	1280	1280	1280	640
	b	0		0	0	0	0	0

* a = reciprocal titre before absorption with strain indicated

b = reciprocal titre with homologous organism after absorption with strain indicated

0 = no agglutination at a dilution of 1 in 20.

Table 46

Agglutination reactions with antisera raised to *A. suis* NCTC10841 and P393 and to *A. equuli* ERS29 before and after absorption with heated cells of each immunising strain

Antiserum to strain	Absorbing antigen to strain	Agglutinable antigen to strain:- NCTC10841	P393	ERS29
A. suis NCTC10841	Unabsorbed	1280	640	640
	NCTC10841	0	0	0
	P393	0	0	0
	ERS29	0	0	0
A. suis P393	Unabsorbed	640	1280	640
	NCTC10841	0	0	0
	P393	0	0	0
	ERS29	0	20	0
A. equuli ERS29	Unabsorbed	2560	1280	2560
	NCTC10841	0	0	160
	P393	0	0	320
	ERS29	0	0	0

0 indicates no agglutination at a dilution of 1 in 20.

Table 47

Passive haemagglutination reactions with antisera NCTC10841 and P393 before and after absorption with heat extracts of 3 strains each of *A. suis* and *A. equuli*.

Antiserum to strain		Sensitising and absorbing antigens to strain					
		A. suis			*A. equuli*		
		NCTC 10841	P393	36659 /73	EQ13	ERS29	DRV55
A. suis NCTC10841	a*	20480	20480	10240	10240	10240	10240
	b	0	0	0	0	0	0
A. suis P393	a	10240	20480	20480	20480	20480	20480
	b	0	0	0	0	0	0

a = reciprocal titre before absorption with antigen indicated

b = reciprocal titre with homologous antigen after absorption with antigen indicated

0 = no haemagglutination at a dilution of 1 in 40.

Table 48

The effect of inhibition with heat extracts of two strains each of A. equuli and A. suis which cross-reacted in passive haemagglutination test on the activity of antiserum raised against A. equuli ERS29 heated vaccine

Sensitising antigen to strain	Inhibiting antigen to strain			
	10841	P393	ERS29	RDV55
NCTC10841 (20480)	80*	40	40	40
P393 (40960)	40	20	20	20
ERS29 (20480)	40	20	20	20
RDV55 (20480)	160	80	80	80

* = the reciprocal inhibition titre of antigen indicated.

Figures in parentheses indicate the reciprocal titre in the passive haemagglutination test with with antigen indicated.

O-groups of A. equuli and of the 13 strains of A. lignieresi. Similarly, all the antisera prepared against the heated vaccine of strains of A. equuli (28 A. equuli O group antisera) were also tested against the heated slide antigens of the 16 strains of A. suis and of the 13 strains of A. lignieresi, and two A. lignieresi antisera were tested against the 28 O-group strains of A. equuli and the 13 strains of A. suis. The results obtained with these tests are summarised in Tables 49, 50 and 51. It can be seen from Table 49 that no strains of A. equuli reacted against antisera to A. lignieresi and that only one strain ERS29 (A. equuli O group 28) showed strong positive reaction against 2 A. suis antisera, while the others failed to react against them. Each of the heated slide antigens of the 16 strains of A. suis gave strong positive reactions against not only 2 A. suis antisera but antiserum ERS29 of A. equuli O group 28 (Table 51). Six strains (27585/72, 927/73, 928/73, 18808/73, 18809/73 and 36659/73) reacted even against antiserum ERS12 of A. equuli O group 23; these agglutination patterns are markedly similar to those of the majority of O group 28 of A. equuli. No other antisera, however, appeared to react against any one of the A. suis strains (Table 50). The results obtained with 13 A. lignieresi strains using the 32 antisera in the work are shown in Table 51, from which it can be seen that each of the heated slide antigens of

Table 49

Agglutination reaction in slide tests between 4 antisera prepared against 2 strains each of A. suis and A. lignieresi and 28 A. equuli strains of different O group

Heated slide antigen to strain	O group	Antiserum prepared against strain			
		A. suis		A. lignieresi	
		NCTC10841	P393	A5	A7
RDV1	1	-	-	-	-
RDV28	2	-	-	-	-
RDV45	3	-	-	-	-
RDV62	4	-	-	-	-
ERS3a	5	-	-	-	-
ERS18	6	-	-	-	-
ERS20	7	-	-	-	-
ERS22	8	-	-	-	-
ERS54	9	-	-	-	-
ERS31	10	-	-	-	-
ERS37	11	-	-	-	-
ERS40	12	-	-	-	-
ERS46	13	-	-	-	-
ERS47b	14	-	-	-	-
ERS53	15	-	-	-	-
EQ2	16	-	-	-	-
EQ3	17	-	-	-	-
EQ7	18	-	-	-	-
NCTC3365	19	-	-	-	-
NCTC8529	20	-	-	-	-
NCTC8644	21	-	-	-	-
NCTC8987	22	-	-	-	-
ERS12	23	-	-	-	-
EQ4	24	-	-	-	-
RDV9	25	-	-	-	-
RDV64	26	-	-	-	-
RDV44	27	-	-	-	-
ERS29	28	+	+	-	-

Table 50

Agglutination reactions in slide tests between 32 antisera prepared against strains of 3 species of Actinobacillus and 16 A. suis antigens

Antigen to strain	Antiserum against strain				
	A. suis		A. equuli		28 other antisera
	NCTC 10841	P393	ERS29	ERS12	
NCTC10841	+	+	+	-	-
NCTC10843	+	+	+	-	-
1688/72	+	+	+	-	-
27585/72	+	+	+	+	-
927/73	+	+	+	+	-
928/73	+	+	+	+	-
15296/73	+	+	+	-	-
18808/73	+	+	+	+	-
18809/73	+	+	+	+	-
32452/73	+	+	+	-	-
36659/73	+	+	+	+	-
37558/73	+	+	+	-	-
4015/74	+	+	+	-	-
P247	+	+	+	-	-
P380	+	+	+	+	-
P393	+	+	+	-	-

Table 51

Agglutination reactions in slide tests between 32 antisera prepared against strains of 3 species of Actinobacillus and 13 A. lignieresi antigens

Antigen to strain	Antiserum against strain				28 other antisera
	A. lignieresi		A. equuli		
	A5	A7	RDV1	NCTC8644	
A2	-	-	-	-	-
A3	-	-	-	-	-
A5	+	+	-	+	-
A6	+	+	+	-	-
A7	+	+	+	+	-
A13	-	-	-	-	-
A14	-	-	-	-	-
A17	+	-	-	-	-
A20	+	+	+	-	-
A43	-	-	-	-	-
A49	-	-	-	-	-
A53	+	+	-	+	-
A100	-	-	-	-	-

A. lignieresi has failed to react not only against any of the A. suis antisera but against all A. equuli antisera with the exception of two. Each of these two antisera (RDV1 and NCTC8644) gave positive reactions against 3 strains of A. lignieresi indicating that there is some antigenic relationship between A. equuli and A. lignieresi.

To determine the antigenic relationship between the 3 species of Actinobacillus studied in the work, two strains each of A. equuli, A. suis and A. lignieresi were tested against antiserum raised to A. equuli NCTC3365 heated cells using the immunodiffusion precipitin reaction. This antiserum is known to have agglutinin(s) capable of reacting against common antigenic fraction(s) of all the strains of A. equuli in the work (see Appendix D). In addition to the 6 strains of actinobacilli, two strains each of Pasteurella multocida and Pasteurella haemolytica were included in the test mainly because the genus Pasteurella has been considered by Johnson and Sneath (1973) and Smith (1974) to be closely associated with the genus Actinobacillus. As shown in Plate 34, at least one antigenic determinant in heat-stable antigenic complexes appeared to be shared by all three species of Actinobacillus, A. equuli, A. suis and A. lignieresi. Moreover, it is of interest to note that this common antigen is also shared by P. haemolytica but not by P. multocida.

Plate 34 Immunodiffusion precipitin patterns of 3 species of Actinobacillus and 2 species of Pasteurella against antiserum(as) to A. equuli NCTC3365.

Antigens in outer wells:

A = A. equuli NCTC3365,
E = A. equuli ERS29,
S = A. suis P393,
L = A. lignieresi A17,
M = P. multocida M873/75,
H = P. haemolytica M1012/75,
eq = A. equuli EQ3,
su = A. suis NCTC10841,
li = A. lignieresi A100,
mu = P. multocida M878/75,
ha = P. haemolytica M878/75.

4. DISCUSSION

(a) Morphological and Cultural Characters of A. suis

The study of 16 strains of A. suis has shown that the organisms are similar in morphology, cultural characters and biochemical reactions. In general, the microscopic characteristics and colonial morphology of the strains of A. suis closely resembled those of A. equuli. The morphological characteristics of the organism have been found to vary, due to changes in the culture medium. However, no previous workers have observed such variation in morphology of A. suis in relation to the growth medium. The bizarre forms met with in A. equuli were also observed with all strains of A. suis in the work when the organism was grown on glucose agar. The pleomorphic nature of A. suis was described by all those who studied this organism. The present finding of mostly uniform morphology of A. suis grown on Loeffler's serum and nutrient agar are somewhat different from the results reached by Magnusson (1931) who has observed that the organism grown on Loeffler's serum and in liquid medium was much more varied in its form than that grown on ordinary culture media. However, the same organism when it was grown on blood agar or glucose agar definitely showed a marked pleomorphism.

Although the majority of the previous workers have described the extremely sticky nature of the colonies of

A. suis on primary isolation (Zimmermann, 1964; Bouley, 1966; Mair et al., 1974), Magnusson (1931) has pointed out that the colonies of his strains never became sticky to the same extent as A. equuli. Sato and Sugimura (1955) have also found that the viscous nature of their organisms was much less than that of A. equuli and that no difficulties were encountered in the preparation of agglutinating antigens from their cultures. The sticky growth of A. suis studied in this work is generally less evident than that of A. equuli although the sticky nature of the colonies of A. suis appeared to be more pronounced in relation to the prolonged incubation. Mair et al. (1974) considered that the colonial adherence to agar medium of A. suis is a characteristic feature of freshly isolated strains. They also noted that on continued subcultivation the organism showed little of the colonial adherence to the medium and that the cultures were readily suspended. The slimy growth of the organism in the present work appeared to be more evident if the cultures in liquid medium were kept for 48 to 72 hours, similar findings were reported by Magnusson (1931) who noticed "a rather tough deposit in the bouillon cultures" which had been kept for some time.

(b) Biochemical and fermentative characters of A. suis.

In general, the fermentation of carbohydrates by A. suis appears to be more active than that by A. equuli.

Sixteen of 24 substrates used in the fermentation tests were fermented by all A. suis strains in the work while only four substrates were attacked by all A. equuli strains. The fermentation of arabinose, xylose, dextrose, fructose, galactose, mannose, cellobiose, lactose, maltose, melibiose, sucrose, trehalose, raffinose, dextrin, glycerol and salicin has been observed by the majority of workers who have employed these substrates in their tests (Sato and Sugimura, 1955; Terpstra and Akkermans, 1955; van Dorssen and Jaartsveld, 1962; Ross et al., 1972; Frederiksen, 1973; Mair et al., 1974), but the variation with arabinose (Zimmermann, 1964) and salicin (Magnusson, 1931) has been reported. The non-fermentation of rhamnose, inulin, mannitol, adonitol, sorbitol, dulcitol and inositol observed with all strains of A. suis in the present study has also been noted by all previous workers, with the exception of Magnusson (1931) who recorded fermentation of mannitol.

The biochemical properties of the strains of A. suis in the work appeared to be comparatively uniform. All strains in the present work were catalase positive and this fact was generally agreed by all workers who investigated this activity of the organism (Sato and Sugimura, 1955; Ross et al., 1972; Frederiksen, 1973; Mair et al., 1974). However, van Dorssen and Jaartsveld (1962) have recorded negative results in this test with their strains. No previous workers have examined oxidase

activity of the organism with the exception of Bouley (1966) who recorded positive reaction with his single strain recovered from a sow with acute nephritis. Seven (43.8%) of 16 strains in the work, however, revealed the lack of oxidase activity while the remaining strains showed the evidence of existence of this enzyme. Positive results in the tests for β-galactosidase and urease activities and nitrate reduction seen in the present work with all A. suis strains have been observed by Terpstra and Akkermans (1955), Frederiksen (1973) and Mair et al. (1974). The ability of A. suis to hydrolyse aesculin was considered by Frederiksen (1971, 1973) and Mair et al. (1974) to be a characteristic feature of the organism and the fact is further supported by the present finding where all strains studied gave aesculin-positive reactions. The failure to produce indole and the lack of gelatinase activity of the present strains of A. suis confirmed the findings of all other workers. Similarly, there is almost complete agreement on the reduction of nitrate to nitrite by the organism. The growth of the organism on MacConkey's medium was recorded by Mair et al. (1974) and Cowan (1974) while no growth occurred in citrate medium (van Dorssen and Jaartsveld, 1962; Bouley, 1966; Ross et al., 1972; Mair et al., 1974). Although no previous workers have examined the ability of the organism to hydrolyse sodium hippurate, fifteen (93.8%) of 16 strains showed a positive reaction in the test.

In the present investigation all strains gave negative results in either methyl red or Vöges-Proskauer tests, the fact also observed by Ross et al. (1972) and Mair et al. (1974). However, positive results in methyl red test were recorded by van Dorssen and Jaartsveld (1962) with their strains and Bouley (1966) observed a weak positive reaction in the Vöges-Proskauer test with his single strain.

Different results in the test for the ability of the organism to produce hydrogen sulphide were obtained by different workers, Sato and Sugimura (1955), Zimmermann (1964), Ross et al. (1972) and Cowan (1974) having observed positive results and Magnusson (1931), van Dorssen and Jaartsveld (1962), Frederiksen (1973) and Mair et al. (1974) negative results.

The haemolytic activity of the organism was considered by all previous workers to be the most pronounced character of the organism and this is borne out in the present investigation. All strains of A. suis included in the work showed a marked haemolysis on both sheep blood agar and horse blood agar but the activity is more pronounced on sheep blood agar than horse blood agar.

The production of pigment by A. suis has not been recorded previously. However, the fact that all the strains in the work consistently produced creamy yellow pigment is of interest since it appears to be one of the distinguishing characters of the porcine haemolytic

strains.

(c) Serological properties of A. suis.

The fact that two antisera prepared against strains NCTC10841 and P393 agglutinated every strain of A. suis in the work to a titre comparable to that obtained with their homologous antigens, suggested that, antigenically, all the strains studied are very closely related to each other. This was further shown by almost complete cross-absorption of agglutinins. However, minor antigenic differences existing between strains were noted with the immunodiffusion precipitin test and immunoelectrophoresis. The antigenic structure of A. suis has not been studied by any of the previous workers with the exception of Sato and Sugimura (1955) who have investigated the serological properties of 32 strains of A. suis recovered from pigs with swine fever and concluded that "the organism is of the monophasic type in antigenicity". The present findings of antigenic similarities between strains of A. suis generally confirmed the results reached by Sato and Sugimura (1955).

The results obtained with the serological comparison of five porcine haemolytic strains (A. equuli O group 28) with 16 strains of A. suis suggest that the porcine strains of A. equuli are antigenically identical to those of A. suis. Furthermore, observations made in the serological comparison of horse strains of A. equuli

O group 28 with A. suis indicate that there are marked antigenic similarities between these two groups. Wetmore et al. (1963) have observed considerable serological cross reactions between an haemolytic variant of A. equuli isolated from a foal and five haemolytic strains recovered from irradiated pigs. They remarked upon the marked serological cross reactions between A. equuli and A. lignieresi, and considered that A. equuli and A. lignieresi isolated from various sources intergrade in serological relationships. Furthermore, it has been proposed that the use of the specific taxon equuli be abrogated and that the strains heretofore classified as A. equuli be reclassified as A. lignieresi. So they classified all their haemolytic strains from pigs and a foal as belonging to A. lignieresi. On the other hand, Mráz (1968) took the view that the strains of van Dorssen and Jaartsveld (1962) and those of Wetmore et al. (1963) only represented haemolytic variants of A. equuli. However, the porcine haemolytic strains of Wetmore et al. (1963) are considered to be A. suis by W. Frederiksen of Statens Seruminstitut, Aalborg, Denmark on the basis of their different biochemical characteristics from those of A. equuli and A. lignieresi. One (P380= Wetmore 1384 =*ATCC15559) of the porcine haemolytic strains of Wetmore et al. (1963), which was kindly supplied by Dr. W. Frederiksen, was investigated together with another 15 A. suis strains, and the results do support

* American Type Culture Collection

the view reached by Ross et al. (1972) and Mair et al. (1974) who regarded that A. suis differs sufficiently from both A. equuli and A. lignieresi to warrant its retention as a separate species.

(d) Comparative biochemical, cultural and serological properties of the three species of Actinobacillus

The work reported in this section is intended simply for a preliminary investigation of A. suis in comparison with A. equuli and A. lignieresi. However, the observations made in this study presented useful criteria for the differentiation of these rather taxonomically disputed species of organism. As shown in Table 52, A. suis can be distinguished from A. lignieresi and A. equuli Type I by its haemolytic activity, ability to hydrolyse aesculin and to produce acid from cellobiose and salicin, failure to ferment mannitol and capacity to produce creamy yellow pigment. It is of interest to note that there is a marked similarity in the biochemical properties between A. suis and Type II of A. equuli although minor differences in the fermentation of arabinose, catalase activity and ability to produce pigment were observed between these two groups of organism. The difference between A. lignieresi and Type I of A. equuli is not very pronounced but A. lignieresi can be differentiated from the latter by their inability to hydrolyse sodium hippurate and to form broth deposit and

Table 52

Differential characteristics of the three species of genus Actinobacillus

Character	A. lignieresi	A. equuli		A. suis
		Type I	Type II	
Catalase	+	d	d	+
Aesculin hydrolysis	-	-	+	+
Hippurate hydrolysis	-	+	+	+
Haemolysis of sheep rbc	-	-	+	+
Broth deposit	-	+	+	+
Creamy yellow pigment prodn.	-	-	-	+
Fermentation of Arabinose	-	d	d	+
Cellobiose	-	-	+	+
Lactose	(+)	+	+	+
Melibiose	-	+	+	+
Trehalose	-	+	+	+
Raffinose	-	+	+	+
Mannitol	+	+	-	-
Salicin	-	-	+	+

+ = 85-100 per cent. strains positive
d = 16-94 per cent. strains positive
- = 0-15 per cent. strains positive
() = delayed reaction

by their failure to produce acid from melibiose, trehalose and raffinose. The difference between these two groups in their biochemical properties were also observed in the ability of A. lignieresi to ferment lactose with delayed reaction, but Type I of A. equuli fermented this sugar promptly (within 24 hours).

Comparative serological studies of three species of Actinobacillus (A. equuli, A. suis and A. lignieresi) in the work are merely of a preliminary nature, but the observations made in this study indicate that there is an antigenic relationship between the three species of the genus. Interspecies antigenic relationships between A. equuli and A. suis have been reported by Magnusson (1931), Mráz (1968) and Ross et al. (1972) and that between A. equuli and A. lignieresi by Haupt (1934), Vallée et al. (1963), Phillips (1966) and Mráz (1968). Although the three species of Actinobacillus have been found to be related antigenically to one another by sharing minor common antigen(s), it is of interest to note that Type II strains of A. equuli are very closely related to A. suis.

Mráz (1968) was of the opinion that haemolytic actinobacilli both from pigs and horses only represented haemolytic variants of A. equuli and thus he proposed to retain A. equuli as the species name for these organisms. Similarly, Larsen (1974) took the view, after finding haemolytic actinobacilli resembling those from pigs (A. suis), that the condition for creation of the new

species name A. suis may be discussed, as the pig is not the only host of these organisms. It is somewhat surprising that both Mráz (1968) and Larsen (1974) have disregarded the differences existing between haemolytic and non-haemolytic actinobacilli from horses although they considered that A. equuli is sufficiently different from A. lignieresi. According to the present study, the differences noted between A. lignieresi and non-haemolytic (Type I) A. equuli appeared to be less evident than those observed between haemolytic (Type II) and non-haemolytic (Type I) A. equuli. In his recent taxonomic study of actinobacilli, Frederiksen (1971, 1973) found that haemolytic strains, which included some of the original strains of Terpstra and Akkermans (1955), Wetmore et al. (1963) and Zimmermann (1964), differed from A. equuli and A. lignieresi in producing acid from arabinose, cellobiose, salicin and aesculin, and concluded that they belonged to the species A. suis. Similar results have been reported by Mair et al. (1974) who have described 8 haemolytic actinobacilli from pigs with septicaemia. Although these workers have only examined haemolytic actinobacilli from pigs mostly with septicaemic conditions, their results are remarkably in agreement with the present findings with haemolytic actinobacilli either from pigs or horses (A. equuli Type II).

Of the 42 strains of Type II of A. equuli thirty-three had been recovered from the upper respiratory tract

of normal horses (the majority from the swabs taken from endotracheal tubes during surgical operations). The remaining 9 strains were isolated from pathological processes in horses (ERS8, ERS28, ERS38 and EQ14) and pigs (EQ9, EQ10, EQ11, EQ12 and EQ13) including submaxillary abscess, abortion, mastitis and septicaemia. The five porcine strains recovered from pigs with septicaemia were antigenically and biochemically very closely related, if not identical, to A. suis in the work. Similarly the majority of equine strains of Type II A. equuli appeared to have close resemblances in their antigenic and biochemical properties with those of A. suis strains. More recently the isolation of A. suis from the nostrils of a two year old colt has been reported (Report, 1975).

Further work is required to characterise the equine haemolytic strains, but the results obtained in the present work appear to suggest the not infrequent occurrence in the upper respiratory tract of normal horses of organisms resembling A. suis and the association of such organisms with disease in this host species.

Johnson and Sneath (1973) have considered that the genus Actinobacillus is phenetically very similar to the genera Haemophilus and Pasteurella. Furthermore, the genera Actinobacillus and Pasteurella have so much in common that their fusion has even been suggested (Sneath and Johnson, 1973; Cowan, 1974). It has been

known for many years that A. lignieresi, A. equuli and A. suis are related to P. multocida (Sato and Sugimura, 1955; Sakazaki and Watanabe, 1956; Sneath and Cowan, 1958), and Mráz (1969a,b) has given further evidence of the close similarity of both A and T types of P. haemolytica to A. lignieresi. Even though it is difficult to base conclusions on the results of such a small number of strains, the present finding that P. haemolytica is antigenically associated with actinobacilli further supported the opinions of Smith (1974) that the genus Pasteurella is closely related to the genus Actinobacillus, to which the species P. haemolytica reveals the closest relationship.

GENERAL CONCLUSION

A survey of the literature has shown that Actinobacillus equuli has not been well characterised by previous workers, thus even its identity has been questioned because of its uncertain position of taxonomic status. In order to provide a useful criterion for the identification of the organism, one hundred and thirty-eight strains of A. equuli including 6 NCTC strains have been investigated. The majority of these organisms had been recovered from the respiratory tracts of normal horses and provisionally labelled as A. equuli, and the remainder were isolated in the period of 1962 to 1973 from diseased horses and pigs.

The morphological, cultural, biochemical and fermentative characteristics by which the organism is usually identified have been evaluated. The work has shown that A. equuli is a Gram-negative, non-motile bacillus characterised by a lack of uniformity in morphology, depending upon the culture medium. Although the organism shows no evidence of the presence of a capsule, extracellular slime material of a mucoid nature, which makes broth cultures viscid and colonies sticky and is very difficult to remove from an agar surface, has been demonstrated. As unusual pleomorphic forms simulating those met with in the pleuropneumonia group are seen in the cultures of the organism, it has been thought of interest to find out whether L phase

variation occurs spontaneously with this group of organisms.

A. equuli has been shown to ferment promptly xylose, dextrose, fructose, mannose, galactose, lactose, maltose and sucrose, but does not attack rhamnose, inulin, adonitol, dulcitol and inositol. Fermentation of glycerol usually occurs after 3 days and melibiose, trehalose and raffinose are fermented by the majority of the strains. Differences between strains are observed in the fermentation reactions with arabinose, cellobiose, mannitol, sorbitol and salicin.

The organism grows on MacConkey's medium, but it does not grow both in Koser's citrate medium and KCN broth. It reduces nitrates to nitrites, but fails to produce indole and ammonia. Positive reactions are given in the tests for β-galactosidase, phosphatase and urease activity, while negative results are recorded with the M.R. and V.P. tests. It fails to hydrolyse gelatin and starch, to utilise malonate and to bring about deamination of phenylalanine, but the majority of the strains hydrolyse sodium hippurate.

The haemolytic ability of the organism appears to provide one of the distinguishing features of the strains investigated in the work since some biochemical properties such as the fermentation of mannitol, salicin and cellobiose and hydrolysis of aesculin are closely related to this activity. It has been proved possible

to place each of the strains examined into one of two main groups. One group, which is haemolytic on layered sheep blood agar, ferments cellobiose and salicin and hydrolyses aesculin in 85, 90 and 90 per cent.of cases respectively, while the other group which is non-haemolytic fails to ferment cellobiose and salicin and to hydrolyse aesculin. Haemolytic strains mostly failed to produce acid from mannitol whereas non-haemolytic strains are mannitol fermenters.

Antigenic studies of the organism have shown that all, with the exception of two, of the strains can be arranged into 28 groups on the basis of their heat-stable antigens (O antigens). The classification of no less than 98.6 per cent. of the strains into specific O groups suggests that the major heat-stable antigens concerned with this are quite distinct. The existence of minor antigens which are common to several groups is indicated by the occurrence of cross agglutination reactions between groups. The results obtained with agglutinin-absorption tests provide evidence that there are some antigenic differences even within the same O groups. The commensal and pathological opportunist status of A. equuli is further supported by the fact that none of the identified multiple serogroups of the organism is consistently associated with diseases in horses.

The results obtained with living and formolised antigens of A. equuli have shown that these antigens

differ serologically from those treated with heat. The heat-labile antigens appear to be associated with the extracellular slime and are common to organisms of different O groups. That the complete blocking of somatic (O) agglutination by heat-labile antigens is observed if only the antigens prepared from rich slime producers were employed, suggests that the heat-labile antigen is located superficially on the bacterial cell in the form of extracellular slime which is not necessarily complete and that the slime is antigenic.

Agglutination tests offer a means of classifying this serologically heterogeneous organism, but the autoagglutinability of rough strains and inagglutinability of the organism owing to its viscous nature have often been found to interfere with such tests. Thus the feasibility of applying other serological procedures to overcome the difficulties has been sought and a passive haemagglutination test for the detection of antibodies to A. equuli was developed, using sheep erythrocytes modified with A. equuli heat extracts. The conditions for the passive haemagglutination test have been studied and are discussed in some detail with special reference to the soluble antigens of A. equuli. The extreme antigenic diversity of A. equuli has often been considered to be too great to allow for the use of a diagnostic serological test, but the work has shown that the potential usefulness of the polyvalent haemagglutination test for the

serological diagnosis of the disease would overcome the difficulties incurred by the serological heterogeneity of the organism. The passive haemagglutination test developed has been proved to be a useful tool in the study of the antigenic structure of A. equuli and that its advantage over the other serological procedures is its specificity and sensitivity.

The serological behaviour of the heat-stable antigens was compared by the use of immunodiffusion precipitin tests and immunoelectrophoresis. Immunodiffusion precipitin reactions of a partial identity were obtained with the heat extract and the Westphal-type lipopolysaccharide. A noticeable spur with these antigens indicated the presence of an additional antigenic determinant in the Westphal-type lipopolysaccharide that was not present in the heat extract. Phenol treatment of the heat extract resulted in the formation of lines of complete identity with both the Westphal-type lipopolysaccharide and with heated Westphal-type lipopolysaccharide, suggesting that phenol has the ability to break down the heat stable antigen (O antigen) into smaller sub-units. Heat extracts of the organisms were used in the immunodiffusion precipitin test to group A. equuli strains into 28 O-groups. The test appeared to be the test of choice for the serogrouping of isolates of A. equuli because of its specificity and its simplicity and rapidity in performance. Moreover, the immunodiffusion precipitin

test has a great advantage over the other serological methods in showing a clear proof of an antigenic relationship between O groups. All the strains in the work have shown themselves to share at least one common heat-stable antigen which could be demonstrated by means of the immunodiffusion precipitin test. That there was not reciprocity of the cross reaction in every case suggests the presence of common hapten(s) rather than complete antigen(s) in the common somatic component(s) of the organism.

The O groups of A. equuli presented in the work are based upon an antigenic complex which may be analogous to that of the O groups of Salmonella organisms. The work has shown that such a classification is fairly well correlated with certain biochemical characters of the organism and the majority of the strains can be grouped into two main divisions. The term 'biotype' is applied for the differentiation of these divisions of A. equuli. Of 138 strains studied, 84 were of Type I, 42 were of Type II and the remaining 12 were intermediate in type. Type I strains gave a positive reaction with mannitol, whilst their reaction with salicin and cellobiose were negative until the 14th day. All the strains comprising this Type were aesculin-negative and, with the exception of only one, non-haemolytic. These strains fell into O groups 1 - 22 inclusive. On the other hand, Type II strains were mannitol-negative, whilst the majority

(92.9%) of the strains fermented salicin and cellobiose and hydrolysed aesculin, and all haemolysed sheep erythrocytes. The Type II strains were made up of O groups 24 - 28 inclusive.

The study of 16 strains of Actinobacillus suis has shown that the strains are remarkably similar in their morphology, cultural characters and biochemical properties. All strains of A. suis have also been found to be serologically homogeneous in relation to the agglutination test with the antisera prepared against heated bacterial suspensions, but minor antigenic differences existing between some of the strains were demonstrated with immunodiffusion precipitin tests and immunoelectrophoresis.

An antigenic relationship between A. equuli, A. suis and A. lignieresi has been demonstrated. Comparative biochemical and serological studies of 3 species of Actinobacillus (A. equuli, A. suis and A. lignieresi) have shown that A. suis is closely related to Type II of A. equuli and that A. suis differs sufficiently from both A. lignieresi and Type I of A. equuli to warrant its separation in a different species within the genus.

Further work is required to characterise haemolytic actinobacilli from horses and pigs, but the results obtained in the work appear to suggest the not infrequent occurrence in the upper respiratory tract of normal horses of organisms resembling A. suis and the

association of such organisms with disease in this host species on occasions.

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APPENDIX A: Sources and fermentative and biochemical properties of strains of Actinobacillus equuli examined in the present study.

All the organisms included in this appendix gave the reactions listed below with the following tests and substrates:-

Ammonia	-	Citrate	-	Gelatin(stab)	-
Gluconate	-	Indole	-	Malonate	-
M.R.	-	ONPG	+	Phenylalanine	-
Phosphatase	+	Urease	+		
Rhamnose	-	Dextrose	+	Fructose	+
Lactose	+	Sucrose	+	Inulin	-
Adonitol	-	Dulcitol	-	Inositol	-

The biochemical and fermentative reactions for any one strain are given on two consecutive pages.

Key:

Fermentative activities:
- \+ = fermentation with acid but no gas within 24 hours.
- +2, +5 = fermentation after 2, 5 days.
- \- = no fermentation within 14 days.

Biochemical tests:
- \+ = positive reaction.
- ± = weak positive reaction.
- (+) = late positive reaction.
- \- = negative reaction.
- α = partial haemolysis.
- β = complete haemolysis.

Strain no.	Host	Site of isolation	Fermentation reactions															
			Arabinose	Xylose	Galactose	Mannose	Cellobiose	Maltose	Melibiose	Trehalose	Raffinose	Dextrin	Starch	Glycerol	Mannitol	Sorbitol	Salicin
RDV1	Exmoor	Endotracheal tube	-	+	+	+	-	+	-	-	-	+	+3	+	+	-	-
RDV2	Exmoor	Endotracheal tube	-	+	+	+	-	+	-	-	-	+	+2	+2	+	-	-
RDV3	Exmoor	Endotracheal tube	-	+	+5	+	-	+	-	-	-	+	-	+3	+	-	-
RDV4	Exmoor	Endotracheal tube	-	+	+	+	+	+	+2	+	+	+	-	+3	-	-	+
RDV5	Exmoor	Endotracheal tube	-	+	+	+	-	+	-	-	-	+	-	+	+	-	-
RDV6	Thoroughbred	Endotracheal tube	-	+	+	+	-	+2	+2	+	+	+	-	+3	+	+2	-
RDV7	Thoroughbred	Endotracheal tube	-	+	+	+2	+	+	+2	+	+	+	-	+2	-	-	+
RDV8	Thoroughbred	Endotracheal tube	-	+	+	+	+	+	+2	+	+	+	-	+2	-	-	+
RDV9	Thoroughbred	Endotracheal tube	-	+	+	+	+	+	+2	+	+	+	-	+3	-	-	+
RDV10	Thoroughbred	Endotracheal tube	-	+	+	+	+3	+2	+3	+	+	+	+3	+3	-	-	+

Biochemical reactions

Strain no.	Catalase	Oxidase	Aesculin hydrolysis	Gelatin(plate) hydrolysis	Hippurate hydrolysis	H_2S production	Growth in KCN broth	Growth on MacConkey	Methylene blue reduction	Nitrate reduction	Starch from glucose	Starch from maltose	Starch hydrolysis	V.P.	Haemolysis of horse rbc	Haemolysis of sheep rbc
RDV1	+	+	-	-	-	+	-	+	+	+	-	+	-	-	-	-
RDV2	+	+	-	-	-	+	-	+	+	+	+|-	+|-	-	-	-	-
RDV3	+	+	-	-	-	+	-	+	-	+	-	+	-	-	-	-
RDV4	-	(+)	+	-	+	-	-	+	-	+	-	-	-	-	α	β
RDV5	+	+	-	-	-	+	-	+	+|-	+	+|-	+|-	-	-	-	-
RDV6	-	(+)	-	-	+	-	-	+	+	+	+|-	+|-	-	-	-	-
RDV7	-	(+)	+	-	+	-	-	+	+	+	-	-	-	+	α	β
RDV8	+	(+)	+	-	+	+	-	+	-	+	+|-	-	-	-	-	α
RDV9	+	+	+	-	-	-	-	+	+|-	+	-	-	-	-	-	α
RDV10	-	+	+	-	-	-	-	+	+|-	+	-	-	-	-	-	β

Strain no.	Host	Site of isolation	Fermentation reactions														
			Arabinose	Xylose	Galactose	Mannose	Cellobiose	Maltose	Melibiose	Trehalose	Raffinose	Dextrin	Starch	Glycerol	Mannitol	Sorbitol	Salicin
RDV11	Thoroughbred	Endotracheal tube	-	+	+	+	-	+	+	+	+	+	-	+5	+	-	-
RDV12	Thoroughbred	Endotracheal tube	-	+	+	+	+	+	+	+	+	+	+4	+2	-	-	+
RDV13	Thoroughbred	Endotracheal tube	-	+	+	+	+	+	+	+	+	+	+7	+2	-	-	+
RDV14	Thoroughbred	Endotracheal tube	-	+	+	+	+2	+	+	+	+	+	-	+3	-	-	+
RDV15	Thoroughbred	Endotracheal tube	+5	+	+	+	-	+	+	+	+	+	+3	+2	+	+	-
RDV16	Thoroughbred	Endotracheal tube	+3	+	+	+3	+2	+	+	+	+	+	-	+3	-	-	+
RDV17	Thoroughbred	Endotracheal tube	+	+	+	+2	+2	+	+	+	+	+	-	+3	-	-	+
RDV18	Thoroughbred	Endotracheal tube	+2	+	+	+2	+5	+	+	+	+	+	+5	+3	-	-	+
RDV19	Thoroughbred	Endotracheal tube	+3	+	+	+2	+	+	+	+	+	+	+5	+5	-	-	+
RDV20	Trotter	Endotracheal tube	+	+	+2	+2	-	+	+	+	+	+	-	+5	+	-	-

Biochemical reactions

Strain no.	Catalase	Oxidase	Aesculin hydrolysis	Gelatin(plate) hydrolysis	Hippurate hydrolysis	H_2S production	Growth in KCN broth	Growth on MacConkey	Methylene blue reduction	Nitrate reduction	Starch from glucose	Starch from maltose	Starch Hydrolysis	V.P.	Haemolysis of horse rbc	Haemolysis of sheep rbc
RDV11	+	(+)	-	-	+	-	-	+	+	+	-	-	-	-	-	-
RDV12	+	+	+	-	+	-	-	+	+	+	-	-	-	-	-	α
RDV13	+	(+)	+	-	+	-	-	+	+	+	+/-	+/-	-	-	-	α
RDV14	-	(+)	+	-	+	-	-	+	+/-	+	-	-	-	-	-	α
RDV15	-	(+)	-	-	+	-	-	+	+	+	-	-	-	-	-	-
RDV16	-	+	+	-	+	+	-	+	+	+	-	-	-	-	β	β
RDV17	-	+	+	-	+	+	-	+	+	+	-	-	-	-	β	β
RDV18	-	+	+	-	+	+	-	+	+	+	-	-	-	-	-	α
RDV19	-	+	+	-	+	+	-	+	+	+	-	-	-	-	-	α
RDV20	+	(+)	-	-	+	-	-	+	+/-	+	+/-	+/-	-	-	-	-

Strain no.	Host	Site of isolation	Fermentation reactions															
			Arabinose	Xylose	Galactose	Mannose	Cellobiose	Maltose	Melibiose	Trehalose	Raffinose	Dextrin	Starch	Glycerol	Mannitol	Sorbitol	Salicin
RDV21	Thoroughbred	Endotracheal tube	+5	+	+	+	-	+	+	+	+	+	+7	+3	+	-	-
RDV22	Shetland	Endotracheal tube	+7	+	+2	+2	-	+	+	+	+	+	-	+2	+	+	-
RDV23	Shetland	Endotracheal tube	+2	+	+2	+2	-	+	+	+	+	+	+5	+2	+	+	+7
RDV24	Shetland	Endotracheal tube	+3	+	+3	+3	-	+	+2	+	+	+	-	+2	+	+	+7
RDV25	Shetland	Endotracheal tube	+5	+	+	+3	-	+	+	+	+	+	+5	+2	+	+	-
RDV26	Shetland	Endotracheal tube	-	+	+	+3	-	+2	+2	+	+	+	-	+2	+	+	-
RDV27	Shetland	Endotracheal tube	-	+3	+2	-	-	+	+2	+2	+2	+	-	+5	+	+	-
RDV28	Pony	Endotracheal tube	+	+	+	+	-	+2	+2	+	+	+	-	+2	+	-	-
RDV29	Pony	Endotracheal tube	+	+	+	+	-	+	+	+	+	+	+5	+2	+	-	-
RDV30	Pony	Endotracheal tube	+	+	+	+	-	+2	+	+	+	+	-	+	+	-	-

Biochemical reactions

Strain no:	Catalase	Oxidase	Aesculin hydrolysis	Gelatin(plate) hydrolysis	Hippurate hydrolysis	H_2S production	Growth in KCN broth	Growth on MacConkey	Methylene blue reduction	Nitrate reduction	Starch from glucose	Starch from maltose	Starch hydrolysis	V.P.	Haemolysis of horse rbc	Haemolysis of sheep rbc
RDV21	-	(+)	-	-	+	-	-	+	+	+	-	±	-	-	-	-
RDV22	-	+	+	-	+	-	-	+	+	+	-	-	-	-	α	α
RDV23	-	+	+	-	+	-	-	+	+	+	-	-	-	-	α	α
RDV24	-	(+)	-	-	+	-	-	+	+	+	-	-	-	-	β	α
RDV25	-	(+)	+	-	+	-	-	+	+	+	-	-	-	-	-	-
RDV26	-	(+)	-	-	+	-	-	+	+	+	-	-	-	-	-	-
RDV27	-	(+)	-	-	+	+	-	+	±	+	-	-	-	-	-	-
RDV28	-	(+)	-	-	+	-	-	+	±	+	-	-	-	-	-	-
RDV29	-	(+)	-	-	+	-	-	+	±	+	-	-	-	-	-	-
RDV30	-	(+)	-	-	+	-	-	+	+	+	-	-	-	-	-	-

Strain no.	Host	Site of isolation	Fermentation reactions															
			Arabinose	Xylose	Galactose	Mannose	Cellobiose	Maltose	Melibiose	Trehalose	Raffinose	Dextrin	Starch	Glycerol	Mannitol	Sorbitol	Salicin
RDV31	Pony	Endotracheal tube	+	+	+	-	-	+2	+	+	+2	+2	-	+3	+	-	-
RDV32	Pony	Endotracheal tube	+3	+	+	+3	-	+2	+2	+	+2	+3	-	+3	+	-	-
RDV33	Pony	Endotracheal tube	+3	+	+	+	-	+	+2	+	+	+	-	+3	+	-	-
RDV34	Thoroughbred	Endotracheal tube	+	+	+	+3	-	+	+	+	+	+2	-	+5	+2	-	-
RDV35	Garron	Endotracheal tube	+3	+	+	+	+	+2	+	+	+	+	-	+	-	-	+
RDV36	Garron	Endotracheal tube	+3	+	+	+2	+3	+	+	+	+	+	-	+	-	-	+
RDV37	Garron	Endotracheal tube	+3	+	+	+2	+	+	+	+	+	+	-	+3	-	-	+
RDV38	Fell	Endotracheal tube	-	+	+	-	+5	+	+2	+	+	+3	-	+3	-	-	+
RDV39	Fell	Endotracheal tube	+3	+	+	-	+3	+2	+3	+	+	+2	-	+3	-	-	+
RDV40	Fell	Endotracheal tube	-	+	+	-	+2	+2	+	+	+	+	-	-	-	-	+

Biochemical reactions

Strain no.	Catalase	Oxidase	Aesculin hydrolysis	Gelatin(plate) hydrolysis	Hippurate hydrolysis	H_2S production	Growth in KCN broth	Growth on MacConkey	Methylene blue reduction	Nitrate reduction	Starch from glucose	Starch from maltose	Starch hydrolysis	V.P.	Haemolysis of horse rbc	Haemolysis of sheep rbc
RDV31	+	(+)	-	-	+	-	-	+	+	+	-	-	-	-	-	-
RDV32	+	-	-	-	+	-	-	+	+	+	-	-	-	-	-	-
RDV33	+	(+)	-	-	+	-	-	+	+	+	-	-	-	-	-	-
RDV34	+	(+)	-	-	+	-	-	+	+	+	-	-	-	-	-	-
RDV35	+	(+)	+	-	+	-	-	+	-	+	-	±	-	-	-	α
RDV36	-	(+)	+	-	+	-	-	+	±	+	-	-	-	-	α	α
RDV37	-	(+)	+	-	+	-	-	+	-	+	-	-	-	-	-	α
RDV38	-	-	+	-	+	-	-	+	-	+	-	-	-	-	-	α
RDV39	-	-	+	-	+	-	-	+	±	+	-	-	-	-	β	β
RDV40	-	-	+	-	+	-	-	+	±	+	-	-	-	-	α	α

Strain no.	Host	Site of isolation	Fermentation reactions															
			Arabinose	Xylose	Galactose	Mannose	Cellobiose	Maltose	Melibiose	Trehalose	Raffinose	Dextrin	Starch	Glycerol	Mannitol	Sorbitol	Salicin
RDV41	Fell	Endotracheal tube	-	+	+	+	+3	+	+	+	+	+	+7	+3	-	-	+
RDV42	Exmoor	Endotracheal tube	-	+	+	+3	+	+2	+	+	+	+	-	+5	-	-	+
RDV43	Exmoor	Endotracheal tube	-	+	+	+2	+	+	+	+	+	+	-	+3	-	-	+
RDV44	Exmoor	Endotracheal tube	+3	+	+	+	+	+2	+	+	+	+	-	+2	-	-	+
RDV45	Pony	Endotracheal tube	-	+	+	+3	-	+	+	+	+	+	-	+5	+	-	-
RDV46	Pony	Endotracheal tube	-	+	+	+5	-	+	+	+	+	+	-	-	+	-	-
RDV50	Pony	Endotracheal tube	-	+	+	+2	+	+	+	+	+	+	-	+	-	-	+
RDV51	Pony	Endotracheal tube	-	+	+	+3	+	+	+	+	+	+	-	+5	-	-	+
RDV52	Pony	Endotracheal tube	+5	+	+	+	-	+3	+2	+	+	+5	-	+3	+	-	-
RDV53	Pony	Endotracheal tube	+5	+	+	+2	-	+	+	+	+	+7	-	+3	+2	-	-

Biochemical reactions

Strain no.	Catalase	Oxidase	Aesculin hydrolysis	Gelatin(plate) hydrolysis	Hippurate hydrolysis	H_2S production	Growth in KCN broth	Growth on MacConkey	Methylene blue reduction	Nitrate reduction	Starch from glucose	Starch from maltose	Starch Hydrolysis	V.P.	Haemolysis of horse rbc	Haemolysis of sheep rbc
RDV41	-	-	+	-	+	+	-	+	+	+	-	-	-	-	β	β
RDV42	-	(+)	+	-	+	-	-	+	+	+	-	+	-	-	-	β
RDV43	-	(+)	+	-	+	-	-	+	+	+	-	+	-	-	-	β
RDV44	-	(+)	+	-	+	-	-	+	+	+	-	+	-	-	-	β
RDV45	-	-	-	-	+	+	-	+	+|	+	+|	-	-	-	-
RDV46	+	-	-	-	+	+	-	+	-	+	-	-	-	-	-	-
RDV50	-	-	+	-	+	-	-	+	+	+	-	-	-	-	-	β
RDV51	-	-	+	-	+	-	-	+	-	+	-	-	-	-	-	β
RDV52	+	(+)	-	-	+	-	-	+	+	+	-	+|	-	-	-	β
RDV53	-	+	-	-	+	+	-	+	+	+	-	+|	-	-	-	β

Strain no.	Host	Site of isolation	Fermentation reactions															
			Arabinose	Xylose	Galactose	Mannose	Cellobiose	Maltose	Melibiose	Trehalose	Raffinose	Dextrin	Starch	Glycerol	Mannitol	Sorbitol	Salicin
RDV54	Thoroughbred	Endotracheal tube	-	+	+	+2	-	+	+	+2	+	+	-	+3	-	-	-
RDV55	Thoroughbred	Endotracheal tube	-	+	+	+	-	+	+	+	+	+	-	+2	-	-	-
RDV56	Pony	Endotracheal tube	-	+2	+	-	+5	+	+2	+	+3	+	-	+7	-	+	+
RDV59	Thoroughbred	Endotracheal tube	+2	+	+	+2	+	+	+	+	+	+	-	+2	-	-	+
RDV60	Thoroughbred	Endotracheal tube	-	+2	+	+2	-	+	+	+	+	+2	-	+2	+	-	-
RDV61	Thoroughbred	Endotracheal tube	-	+	+	+2	-	+	+	+2	+	+	-	+5	+	-	-
RDV62	Thoroughbred	Endotracheal tube	-	+	+	+2	-	+2	+	+	+	+2	-	+5	+	-	-
RDV63	Thoroughbred	Endotracheal tube	-	+	+	+3	-	+	+2	+	+	+	-	+5	+2	-	-
RDV64	Thoroughbred	Endotracheal tube	-	+2	+	+2	+3	+	+	+	+	+3	-	-	-	-	+
RDV65	Thoroughbred	Endotracheal tube	-	+	+	+3	+2	+	+	+	+	+	-	+3	-	-	+

Biochemical reactions

Strain no.	Catalase	Oxidase	Aesculin hydrolysis	Gelatin(plate) hydrolysis	Hippurate hydrolysis	H_2S production	Growth in KCN broth	Growth on MacConkey	Methylene blue reduction	Nitrate reduction	Starch from glucose	Starch from Maltose	Starch hydrolysis	V.P.	Haemolysis of horse rbc	Haemolysis of sheep rbc
RDV54	-	-	-	-	+	-	-	+	+	+	-	-	-	-	α	α
RDV55	-	+	-	-	+	+	-	+	+	+	-	-	-	-	α	α
RDV56	-	(+)	+	-	+	+	-	+	+	+	-	-	-	-	α	β
RDV59	-	(+)	+	-	+	+	-	+	±	+	-	-	-	-	α	α
RDV60	-	(+)	-	-	+	+	-	+	+	+	-	±	-	-	-	-
RDV61	-	(+)	-	-	+	+	-	+	+	+	-	±	-	-	-	-
RDV62	-	(+)	-	-	+	-	-	+	+	+	-	±	-	-	-	-
RDV63	-	(+)	-	-	+	-	-	+	-	+	-	+	-	-	-	-
RDV64	-	(+)	+	-	+	-	-	+	±	+	-	-	-	-	α	β
RDV65	-	(+)	+	-	+	-	-	+	+	+	-	-	-	-	α	β

Strain no.	Host	Site of isolation	Fermentation reactions															
			Arabinose	Xylose	Galactose	Mannose	Cellobiose	Maltose	Melibiose	Trehalose	Raffinose	Dextrin	Starch	Glycerol	Mannitol	Sorbitol	Salicin
ERS1	Horse	Submaxillary abscess	-	+	+	+	-	+	-	-	+2	+	-	+2	+	-	-
ERS2	Foal		-	+	+	+	-	+	+2	-	+	+	-	+	+	+	-
ERS3a	Foal	Liver	-	+	+	-	-	+	+	+	+	+3	-	+2	+	-	-
ERS3b	Foal	Navel	-	+2	+2	+3	-	+	+	+2	+	+3	+7	+5	+	-	-
ERS4	Foal	Nasal discharge	-	+2	+	+2	-	+	+2	+	+	+	-	+3	+	+	-
ERS5			-	-	+	+	-	-	+3	+	+	+5	-	+3	+	-	-
ERS6		Endotracheal tube	-	+2	+	+2	+2	+	+3	+	+	+	+5	+3	-	-	+
ERS7	Horse	Mouth	-	+	+	+3	+	+	+3	+	+	+	-	+3	+	-	+
ERS8	Horse	Submaxillary abscess	-	+	+	+2	+	+	+2	+	+	+3	-	+3	-	-	+
ERS10	Horse	Mucus from endotracheal tube	-	+	+	+2	-	+	+3	+	+	+	+7	+5	+	+	+7

Biochemical reactions

Strain no.	Catalase	Oxidase	Aesculin hydrolysis	Gelatin(plate) hydrolysis	Hippurate hydrolysis	H_2S production	Growth in KCN broth	Growth on MacCockey	Methylene blue reduction	Nitrate reduction	Starch from glucose	Starch from maltose	Starch hydrolysis	V.P.	Haemolysis of horse rbc	Haemolysis of sheep rbc
ERS1	+	(+)	-	-	-	+	-	+	+	+	-	±	-	+	-	-
ERS2	-	-	-	-	+	-	-	+	+	+	-	-	-	-	-	-
ERS3a	-	+	-	-	+	-	-	+	+	+	-	-	+	-	-	-
ERS3b	-	(+)	-	-	+	-	-	+	-	+	-	-	+	-	-	-
ERS4	-	(+)	-	-	+	-	-	+	+	+	-	-	-	-	-	-
ERS5	-	(+)	-	-	+	-	-	+	+	+	-	-	-	-	-	-
ERS6	-	-	+	-	-	+	-	+	+	+	-	-	-	-	α	α
ERS7	-	-	+	-	+	-	-	+	-	+	-	±	-	-	α	β
ERS8	+	-	+	-	+	+	-	+	+	+	-	-	-	+	α	β
ERS10	-	-	+	-	+	+	-	+	-	+	-	-	-	-	α	β

Strain no.	Host	Site of isolation	Fermentation reactions															
			Arabinose	Xylose	Galactose	Mannose	Cellobiose	Maltose	Melibiose	Trehalose	Raffinose	Dextrin	Starch	Glycerol	Mannitol	Sorbitol	Salicin
ERS11	Colt	Mouth	-	+	+	+5	-	+	+2	+	+	+	-	+2	+	-	-
ERS12	Horse	Pharyngeal swab	+2	+	+	+3	+3	+	+2	+	+	+	-	+3	+	+	+
ERS13	Horse	Endotracheal swab	-	+	+2	+3	+	+	+	+	+	+	-	+3	+	+	+
ERS14	Foal	Submaxillary abscess	-	+	+	+	-	+	-	-	+2	+	-	+	+	-	-
ERS15	Horse		-	+	+	+2	+2	+	+	+	+	+	-	+3	-	-	+
ERS16	Horse	Endotracheal swab	+3	+	+	+	-	+	+	+	+	+	-	+7	+	+	-
ERS17	Horse	Nasal swab	-	+	+2	+3	-	+	+5	+	+	+	-	+2	+	-	-
ERS18	Horse	Endotracheal swab	-	+	+	+2	-	+	+2	+	+	+	-	+3	+	-	-
ERS19	Horse	Mouth swab	-	+	+	+2	-	+	+	+	+	+	-	+3	+	-	-
ERS20	Horse	Wound swab	-	+	+	+2	-	+	+	+	+	+3	-	+2	+	-	-

Biochemical reactions

Strain no.	Catalase	Oxidase	Aesculin hydrolysis	Gelatin(plate) hydrolysis	Hippurate hydrolysis	H_2S production	Growth in KCN broth	Growth on MacConkey	Methylene blue reduction	Nitrate reduction	Starch from glucose	Starch from maltose	Starch hydrolysis	V.P.	Haemolysis of horse rbc	Haemolysis of sheep rbc
ERS11	-	-	-	-	+	-	-	+	+	+	-	+	-	+	-	-
ERS12	-	-	+	-	+	+	-	+	+	+	-	-	-	-	α	β
ERS13	-	-	+	-	+	-	-	+	+	+	-	-	-	-	α	β
ERS14	+	(+)	-	-	-	+	-	+	+	+	+	+	-	+	-	α
ERS15	+	-	+	-	+	-	-	+	+	+	-	-	-	-	α	β
ERS16	+	-	-	-	+	-	-	+	±	+	-	-	-	-	-	-
ERS17	-	-	-	-	+	-	-	+	-	+	-	±	-	-	-	-
ERS18	-	-	-	-	+	-	-	+	+	+	-	+	-	-	-	-
ERS19	-	-	-	-	+	-	-	+	-	+	-	+	-	-	-	-
ERS20	(+)	-	-	-	+	+	-	+	+	+	-	-	-	-	-	-

Strain no.	Host	Site of isolation	Fermentation reactions															
			Arabinose	Xylose	Galactose	Mannose	Cellobiose	Maltose	Melibiose	Trehalose	Raffinose	Dextrin	Starch	Glycerol	Mannitol	Sorbitol	Salicin
ERS21	Horse	Mouth swab	-	+	+2	+3	-	+	+	+	+2	+	-	+2	+	-	-
ERS22	Horse	Endotracheal swab	-	+	+2	+2	-	+2	+	+	+	+	-	+3	+	-	-
ERS23	Horse	Mouth swab	+5	+	+2	+2	-	+3	+2	+	+	+	-	+3	+2	-	-
ERS24	Horse	Mouth swab	-	+	+2	+3	-	+	+2	+	+	+	-	+2	+	-	-
ERS25	Horse	Endotracheal swab	-	+	+	+3	-	+2	+	+	+	+	-	+	+	-	-
ERS26	Horse	Nostril swab	-	+	+	+	-	+	+	+2	+	+	-	+3	+	-	-
ERS27	Horse	Mouth swab	-	+	+2	+3	-	+2	+	+	+	+	-	-	+	-	-
ERS28	Equine Foetus	Chorion	-	+	+	+3	+3	+	+	+	+	+	-	+3	-	-	+
ERS29	Horse	Pharyngeal swab	-	+	+	+2	+	+	+	+	+	+	-	+	-	-	+
ERS30	Horse	Endotracheal swab	-	+	+	+2	-	+	+	+	+	+	-	+	+	-	-

Biochemical reactions

Strain no.	Catalase	Oxidase	Aesculin hydrolysis	Gelatin(plate) hydrolysis	Hippurate hydrolysis	H_2S production	Growth in KCN broth	Growth on MacConkey	Methylene blue reduction	Nitrate reduction	Starch from glucose	Starch from maltose	Starch hydrolysis	V.P.	Haemolysis of horse rbc	Haemolysis of sheep rbc
ERS21	-	(+)	-	-	+	-	-	-	-	+	-	-	-	-	-	-
ERS22	-	(+)	-	-	+	-	-	+	+	+	-	-	-	-	-	-
ERS23	+	-	-	-	+	-	-	+	+	+	-	-	-	-	-	-
ERS24	+	(+)	-	-	+	-	-	+	+	+	-	±	-	-	-	-
ERS25	+	-	-	-	+	-	-	+	+	+	-	±	-	-	-	-
ERS26	-	-	-	-	+	-	-	+	+	+	-	-	-	-	-	-
ERS27	-	(+)	-	-	+	-	-	+	+	+	-	±	-	-	-	-
ERS28	-	(+)	+	-	+	+	-	+	-	+	-	-	-	-	β	-
ERS29	-	(+)	+	-	+	-	-	+	+	+	-	±	-	-	α	-
ERS30	-	-	-	-	+	-	-	+	+	+	-	±	-	-	β	-

Strain no.	Host	Site of isolation	Fermentation reactions															
			Arabinose	Xylose	Galactose	Mannose	Cellobiose	Maltose	Melibiose	Trehalose	Raffinose	Dextrin	Starch	Glycerol	Mannitol	Sorbitol	Salicin
ERS31	Horse	Mouth swab	-	+	+2	+2	-	+	+	+	+	+	-	+2	+2	+	-
ERS32	Horse	Endotracheal swab	-	+	+	+3	-	+	+	+	+2	+	-	+2	+	+	-
ERS33	Horse	Mouth swab	-	+2	+2	+5	-	+	+	+2	+2	+5	-	-	+	+	-
ERS34	Horse	Endotracheal swab	-	+	+3	+3	-	+	+	+	+	+	-	+3	+2	-	-
ERS35	Horse	Endotracheal swab	-	+	+2	+2	-	+	+	+	+	+2	-	+2	+	-	-
ERS36	Horse	Nasal swab	-	+	+2	+2	-	+	+	+	+	+	-	+5	+2	-	-
ERS37	Horse	Endotracheal swab	+3	+	+	+3	-	+	+	+	+	+	-	+3	+2	-	-
ERS38	Foal	Septicaemia	-	+	+	+2	+	+	+	+	+	+	-	+2	-	-	+
ERS39	Horse	Mouth swab	+	+	+	+3	-	+	+	+	+	+	-	-	+	+	-
ERS40	Horse	Endotracheal swab	+2	+	+	+3	-	+	+	+	+	+	-	+5	+	+	-

Biochemical reactions

Strain no.	Catalase	Oxidase	Aesculin hydrolysis	Gelatin(Plate) hydrolysis	Hippurate hydrolysis	H_2S production	Growth in KCN broth	Growth on MacConkey	Methylene blue reduction	Nitrate reduction	Starch from glucose	Starch from maltose	Starch hydrolysis	V.P.	Haemolysis of horse rbc	Haemolysis of sheep rbc
ERS31	-	(+)	-	-	+	-	-	+	+	+	-	-	-	-	-	-
ERS32	+	(+)	-	-	+	-	-	+	+/-	+	-	-	-	-	-	-
ERS33	-	(+)	-	-	+	-	-	+	+/-	+	-	+	-	-	-	-
ERS34	-	(+)	-	-	+	-	-	+	+/-	+	-	+/-	-	-	-	-
ERS35	+	(+)	-	-	+	-	-	+	+/-	+	-	+/-	-	-	-	-
ERS36	-	(+)	-	-	+	-	-	+	+	+	-	-	-	-	-	-
ERS37	-	(+)	-	-	+	-	-	+	-	+	-	-	-	-	-	-
ERS38	-	(+)	+	-	+	-	-	+	+	+	-	-	-	-	-	β
ERS39	-	(+)	-	-	+	-	-	+	+	+	-	-	-	-	-	-
ERS40	-	+	-	-	+	-	-	+	+	+	-	-	-	-	-	-

Strain no.	Host	Site of isolation	Fermentation reactions															
			Arabinose	Xylose	Galactose	Mannose	Cellobiose	Maltose	Melibiose	Trehalose	Raffinose	Dextrin	Starch	Glycerol	Mannitol	Sorbitol	Salicin	
ERS41	Horse	Mouth swab	+2	+	+2	+5	-	+	+2	+	+	+2	-	+5	-	-	-	
ERS42	Horse	Mouth swab	-	+	+2	+5	-	+	+	+	+	+	+7	+3	+	-	-	
ERS43	Horse	Endotracheal tube	-	+	-	-	-	+	+	+2	+	+	-	+3	-	-	-	
ERS44	Horse	Endotracheal tube	-	+	+	+5	-	+	+	+	+	+	+7	+5	+	-	-	
ERS45	Horse	Mouth swab	-	+	+	+2	-	+	+2	+	+	+	-	+7	+	-	-	
ERS46	Horse	Guttural pouch	-	+	+	+3	-	+	+2	+	+	+2	-	+5	+	-	-	
ERS47a	Horse	Submaxillary abscess	+3	+	+2	+5	-	+	+	+	+	+2	-	+3	+	+	-	
ERS47b	Horse	Submaxillary abscess	+5	+	+2	+3	-	+	+	+	+	+2	-	+5	+	+	-	
ERS48a	Horse	Submaxillary abscess	+	+	+	+	-	+	+	+	+	+	-	+2	+	-	-	
ERS48b	Horse	Submaxillary abscess	-	+	+	+5	-	+	+	+	+	+3	-	+3	+	-	-	

Biochemical reactions

Strain no.	Catalase	Oxidase	Aesculin hydrolysis	Gelatin (Plate) hydrolysis	Hippurate hydrolysis	H_2S production	Growth in KCN broth	Growth on MacConkey	Methylene blue reduction	Nitrate reduction	Starch from glucose	Starch from maltose	Starch hydrolysis	V.P.	Haemolysis of horse rbc	Haemolysis of sheep rbc
ERS41	+	(+)	-	-	+	-	-	+	+	+	-	-	-	-	-	-
ERS42	-	(+)	-	-	+	-	-	+	+	+	-	-	-	-	-	-
ERS43	-	-	-	-	+	-	-	+	±	+	-	-	-	-	-	-
ERS44	-	(+)	-	-	+	-	-	+	+	+	-	-	-	-	-	-
ERS45	-	(+)	-	-	+	-	-	+	+	+	-	-	-	-	-	-
ERS46	+	(+)	-	-	+	-	-	+	±	+	-	-	-	-	-	-
ERS47a	-	(+)	-	-	+	-	-	+	+	+	-	-	-	-	-	-
ERS47b	-	(+)	-	-	+	-	-	+	+	+	-	-	-	-	-	-
ERS48a	-	(+)	-	-	+	-	-	+	±	+	-	+	-	-	-	-
ERS48b	-	(+)	-	-	+	+	-	+	+	+	-	±	-	-	-	-

Strain no.	Host	Site of isolation	Fermentation reactions															
			Arabinose	Xylose	Galactose	Mannose	Cellobiose	Maltose	Melibiose	Trehalose	Raffinose	Dextrin	Starch	Glycerol	Mannitol	Sorbitol	Salicin
ERS49a	Horse	Nasal swab	+3	+	+2	+2	-	+	+	+	+	+3	+14	-	+2	-	-
ERS49b	Horse	Nasal swab	+3	+	+2	+3	-	-	-2	+	+	+3	-	+3	+2	-	-
ERS51	Horse	Endotracheal tube	-	+	+	+2	-	+	+	+	+	+3	-	+3	+	-	-
ERS52	Horse	Nostril	+2	+	+	+5	-	+	+	+	+	+	-	+5	+2	-	-
ERS53	Horse	Mesenteric lymph node	-	+	+	+3	-	+	+	+	+	+	-	+	+	-	-
ERS54	Foal	Kidney	+5	+	+2	-	-	+	+2	+	+	+	-	+3	+	-	-
ERS55	Horse	Endotracheal tube	+3	+	+	+	-	+	+	+	+	+	-	+2	-	-	-
ERS56	Horse	Mouth	-	+	+2	+2	-	+	+	+	+	+	-	+3	+	-	-
EQ1	Pig	Heart valve	-	+	+	+	-	+	+	+	+	+	-	-	+	-	-
EQ2	Foal	Septicaemia	+	+	+	+	-	+	+	+	+	+	-	+3	+	-	-

Biochemical reactions

Strain no.	Catalase	Oxidase	Aesculin hydrolysis	Gelatin (plate) hydrolysis	Hippurate hydrolysis	H_2S production	Growth in KCN broth	Growth on MacConkey	Methylene blue reduction	Nitrate reduction	Starch from glucose	Starch from maltose	Starch hydrolysis	V.P.	Haemolysis of horse rbc	Haemolysis of sheep rbc
ERS49a	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-
ERS49b	-	(+)	-	-	+	-	-	+	±	+	-	-	-	-	-	-
ERS51	-	-	-	-	+	-	-	+	+	+	-	±	-	-	-	-
ERS52	+	-	-	-	+	-	-	+	+	+	-	±	-	-	-	-
ERS53	-	-	-	-	+	+	-	+	±	+	-	±	-	-	-	-
ERS54	-	-	-	-	+	-	-	+	±	+	-	-	-	-	-	-
ERS55	+	-	-	-	+	+	-	+	-	+	-	-	-	-	α	α
ERS56	+	-	-	-	+	-	-	+	+	+	-	±	-	-	-	-
EQ1	-	(+)	-	-	+	+	-	+	+	+	-	-	-	-	-	-
EQ2	+	(+)	-	-	+	+	-	+	-	+	-	-	-	-	-	-

Strain no.	Host	Site of isolation	Fermentation reactions															
			Arabinose	Xylose	Galactose	Mannose	Cellobiose	Maltose	Melibiose	Trehalose	Raffinose	Dextrin	Starch	Glycerol	Mannitol	Sorbitol	Salicin
EQ3	Pig	Joint	-	+	+3	+2	-	+	+	+	+	+	+5	+3	+2	-	-
EQ4	Foal		-	+	+	+	+2	+	+	+	+	+	+5	+2	-	-	+
EQ5	Pig		-	+	+	+2	-	+	+	+	+	+	+7	+2	+	-	-
EQ6	Piglet	Endocarditis	-	-	-	-	-	+	+	+	+	+	-	-	+	-	-
EQ7			-	+2	+	+3	-	+	+	+	+	+2	-	+5	+3	+3	-
EQ8	Piglet	Heart valve	-	+	+	+	-	+	+	+	+	+2	+7	+3	+	-	-
EQ9	Pig	Kidney	+	+	+	+	+	+	+	+	+	+	-	+3	-	-	+
EQ10	Pig	Septicaemia	+	+	+	+	+	+	+	+	+	+2	-	+2	-	-	+
EQ11	Pig	Lung	+	+	+	+	+	+	+	+	+	+	-	+3	-	-	+
EQ12	Piglet	Septicaemia	+	+	+	+	+	+	+	+	+	+	-	+2	-	-	+

Biochemical reactions

Strain no.	Catalase	Oxidase	Aesculin hydrolysis	Gelatin(plate) hydrolysis	Hippurate hydrolysis	H_2S production	Growth in KCN broth	Growth on MacConkey	Methylene blue reduction	Nitrate reduction	Starch from glucose	Starch from maltose	Starch hydrolysis	V.P.	Haemolysis of horse rbc	Haemolysis of sheep rbc
EQ3	+	(+)	-	-	+	-	-	+	-	+	-	-	-	-	α	β
EQ4	-	+	+	-	+	-	-	+	-	+	-	-	-	-	α	β
EQ5	-	(+)	-	-	+	-	-	+	+	+	-	-	-	-	α	β
EQ6	-	(+)	-	+	+	-	-	+	+	+	-	-	-	-	α	β
EQ7	-	(+)	-	-	+	-	-	+	±	+	-	-	-	-	α	β
EQ8	-	(+)	-	-	+	-	-	+	-	+	-	-	-	-	α	β
EQ9	+	(+)	+	-	-	-	-	+	-	+	-	-	-	-	α	β
EQ10	-	-	+	-	-	-	-	+	-	-	-	-	-	-	α	β
EQ11	+	(+)	+	-	+	-	-	+	-	+	-	-	-	-	α	β
EQ12	+	(+)	+	-	+	-	-	+	-	+	-	-	-	-	α	β

Strain no.	Host	Site of isolation	Fermentation reactions: Arabinose	Xylose	Galactose	Mannose	Cellobiose	Maltose	Melibiose	Trehalose	Raffinose	Dextrin	Starch	Glycerol	Mannitol	Sorbitol	Salicin
EQ13	Pig	Septicaemia	+	+	+	+	+	+	+	+	+	+	-	+2	-	-	+
EQ14	Horse	Mastitis	-	+	+	+2	+	+	+	+	+	+	+7	+2	-	-	+
NCTC 3365	Foal	Pyaemic nephritis	-	+	+2	-	-	+2	+3	+	+2	+3	-	+2	+2	-	-
NCTC 8529	Foal	Blood	-	+	+2	+3	-	+2	+	+	+	+2	+2	+3	+2	-	-
NCTC 8644	Foal	Kidney abscess	-	+	+2	+	-	+	+	+	+	+2	-	-	+	-	-
NCTC 8794	Foal	Septicaemia	-	+	+	+2	-	+	+	+	+	+	-	-	+	-	-
NCTC 8987	Foal	Septicaemia	+3	+	+	+	-	+	+2	+	+	+	-	+5	+	-	-
NCTC 9435	Foal	Kidney	-	+	-	+3	-	+	+	+	+3	+	-	+3	+	-	-

Biochemical reactions

Strain no.	Catalase	Oxidase	Aesculin hydrolysis	Gelatin(plate) hydrolysis	Hippurate hydrolysis	H_2S production	Growth in KCN broth	Growth on MacConkey	Methylene blue reduction	Nitrate reduction	Starch from glucose	Starch from maltose	Starch hydrolysis	V.P.	Haemolysis of horse rbc	Haemolysis of sheep rbc
EQ13	+	(+)	+	-	+	+	-	+	-	+	-	-	-	-	α	β
EQ14	-	+	+	-	+	+	-	+	-	+	+	-	-	-	-	α
NCTC 3365	-	(+)	-	-	-	-	-	+	+	+	-	-	-	-	-	-
NCTC 8529	-	-	-	-	+	-	-	+	-	+	-	-	+	-	-	-
NCTC 8644	-	+	-	-	+	-	-	+	+	+	-	-	-	-	-	-
NCTC 8794	-	(+)	-	-	+	-	-	+	+	+	-	-	-	-	-	-
NCTC 8987	-	(+)	-	-	+	-	-	+	-	+	-	-	-	-	-	-
NCTC 9435	+	(+)	-	+	+	+	-	+	+	+	-	-	+	-	-	-

APPENDIX B: Results of slide agglutination tests between 138 strains of Actinobacillus equuli and 29 antisera prepared against heated bacterial suspensions

Key:

+ = agglutination within one minute

- = no agglutination within one minute

The results for any one strain are given on two consecutive pages.

Antiserum against strain no:-

Strain No:	RDV 1	RDV 28	RDV 45	RDV 62	ERS 3a	ERS 18	ERS 20	ERS 22	ERS 54	ERS 31	ERS 37	ERS 40	ERS 46	ERS 47b	ERS 53
RDV1	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV2	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV3	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV5	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	Antiserum against strain no:-														
Strain No:	EQ2	EQ3	EQ7	NCTC 3365	NCTC 8529	NCTC 8644	NCTC 8987	ERS 12	EQ4	RDV 9	RDV 64	RDV 44	RDV 55	ERS 29	O group
RDV1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
RDV2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
RDV3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
RDV4	-	-	-	-	-	-	-	-	-	-	+	-	-	-	26
RDV5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
RDV6	-	-	+	-	-	-	-	-	-	-	-	-	-	-	18
RDV7	-	-	-	-	-	-	-	-	-	-	-	+	+	+	28
RDV8	-	-	-	-	-	-	-	-	-	-	-	+	+	+	28
RDV9	-	-	-	-	-	-	-	-	-	+	-	-	-	-	25
RDV10	-	-	-	-	-	-	-	-	-	+	-	-	+	+	25
RDV11	+	-	-	-	-	-	-	-	-	-	-	-	-	-	16
RDV12	-	-	-	-	-	-	-	-	-	-	-	+	+	+	28
RDV13	-	-	-	-	-	-	-	-	-	-	-	+	+	+	28
RDV14	-	-	-	-	-	-	-	+	+	-	-	-	-	-	23
RDV15	+	-	-	-	-	-	-	-	-	-	-	-	-	-	16

	Antiserum against strain no:-														
Strain No:	RDV 1	RDV 28	RDV 45	RDV 62	ERS 3a	ERS 18	ERS 20	ERS 22	ERS 54	ERS 31	ERS 37	ERS 40	ERS 46	ERS 47b	ERS 53
RDV16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV20	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
RDV21	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
RDV22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV27	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV28	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV29	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV30	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-

	Antiserum against strain no:-														
Strain No:	EQ2	EQ3	EQ7	NCTC 3365	NCTC 8529	NCTC 8644	NCTC 8987	ERS 12	EQ4	RDV 9	RDV 64	RDV 44	RDV 55	ERS 29	O group
RDV16	-	-	-	-	-	-	-	+	-	-	-	+	+	+	28
RDV17	-	-	-	-	-	-	-	+	-	-	-	+	+	+	28
RDV18	-	-	-	-	-	-	-	+	-	-	-	+	+	+	28
RDV19	-	-	-	-	-	-	-	+	-	-	-	+	+	+	28
RDV20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	14
RDV21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	13
RDV22	-	-	-	-	-	-	-	+	+	-	-	-	-	-	23
RDV23	-	-	-	-	-	-	-	+	+	-	-	-	-	-	23
RDV24	-	-	-	-	-	-	-	+	+	-	-	-	-	-	23
RDV25	-	-	-	-	-	-	-	+	+	-	-	-	-	-	23
RDV26	-	-	-	-	-	-	-	+	+	-	-	-	-	-	23
RDV27	-	-	-	-	-	-	-	+	+	-	-	-	-	-	23
RDV28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
RDV29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
RDV30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2

Strain No:	Antiserum against strain no:- RDV 1	RDV 28	RDV 45	RDV 62	ERS 3a	ERS 18	ERS 20	ERS 22	ERS 54	ERS 31	ERS 37	ERS 40	ERS 46	ERS 47b	ERS 53
RDV31	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV32	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV33	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
RDV34	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV35	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV36	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV37	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV38	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV39	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV41	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV42	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV43	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV44	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV45	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-

Strain No:	Antiserum against strain no:- EQ2	EQ3	EQ7	NCTC 3365	NCTC 8529	NCTC 8644	NCTC 8987	ERS 12	EQ4	RDV 9	RDV 64	RDV 44	RDV 55	ERS 29	O group
RDV31	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
RDV32	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
RDV33	-	-	-	-	-	-	-	-	-	-	-	-	-	-	11
RDV34	-	-	-	-	+	-	-	-	-	-	-	-	-	-	20
RDV35	-	-	-	-	-	-	-	-	-	-	-	+	+	+	27
RDV36	-	-	-	-	-	-	-	-	-	-	-	+	+	+	27
RDV37	-	-	-	-	-	-	-	-	-	-	-	+	+	+	27
RDV38	-	-	-	-	-	-	-	+	-	-	-	+	+	+	28
RDV39	-	-	-	-	-	-	-	+	-	-	-	+	+	+	28
RDV40	-	-	-	-	-	-	-	+	-	-	-	+	+	+	28
RDV41	-	-	-	-	-	-	-	+	-	-	-	+	+	+	28
RDV42	-	-	-	-	-	-	-	-	-	-	-	+	+	+	27
RDV43	-	-	-	-	-	-	-	-	-	-	-	+	+	+	27
RDV44	-	-	-	-	-	-	-	-	-	-	-	+	+	+	27
RDV45	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3

Antiserum against strain no:-

Strain No:	RDV 1	RDV 28	RDV 45	RDV 62	ERS 3a	ERS 18	ERS 20	ERS 22	ERS 54	ERS 31	ERS 37	ERS 40	ERS 46	ERS 47b	ERS 53
RDV46	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
RDV50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV51	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV52	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
RDV53	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
RDV54	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV55	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV56	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV59	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV60	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV61	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
RDV62	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
RDV63	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
RDV64	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RDV65	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Strain No:	Antiserum against strain no:- EQ2	EQ3	EQ7	NCTC 3365	NCTC 8529	NCTC 8644	NCTC 8987	ERS 12	EQ4	RDV 9	RDV 64	RDV 44	RDV 55	ERS 29	O Group
RDV46	-	-	-	-	-	-	-	-	-	-	-	-	-	-	13
RDV50	-	-	-	-	-	-	-	+	-	-	-	+	+	+	28
RDV51	-	-	-	-	-	-	-	+	-	-	-	+	+	+	28
RDV52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	8
RDV53	-	-	-	-	-	-	-	-	-	-	-	-	-	-	8
RDV54	-	-	-	-	-	-	-	+	-	-	-	+	+	+	28
RDV55	-	-	-	-	-	-	-	+	-	-	-	+	+	+	28
RDV56	-	-	-	-	-	-	-	-	-	-	-	+	+	+	27
RDV59	-	-	-	-	-	-	-	+	+	-	-	-	-	-	23
RDV60	-	-	-	-	-	+	-	-	-	-	-	-	-	-	21
RDV61	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4
RDV62	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4
RDV63	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4
RDV64	-	-	-	-	-	-	-	-	-	+	+	-	-	-	26
RDV65	-	-	-	-	-	-	-	-	-	+	+	-	-	-	26

Antiserum against strain no:-

Strain No:	RDV 1	RDV 28	RDV 45	RDV 62	ERS 3a	ERS 18	ERS 20	ERS 22	ERS 54	ERS 31	ERS 37	ERS 40	ERS 46	ERS 47b	ERS 53
ERS1	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ERS2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ERS3a	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
ERS3b	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
ERS4	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
ERS5	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+
ERS6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ERS7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ERS8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ERS10	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
ERS11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ERS12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ERS13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ERS14	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ERS15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Strain No:	Antiserum against strain no:- EQ2	EQ3	EQ7	NCTC 3365	NCTC 8529	NCTC 8644	NCTC 8987	ERS 12	EQ4	RDV 9	RDV 64	RDV 44	RDV 55	ERS 29	O Group
ERS1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
ERS2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ERS3a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5
ERS3b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5
ERS4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	11
ERS5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	11
ERS6	-	-	-	-	-	-	-	+	-	-	-	+	+	+	28
ERS7	-	-	-	-	-	-	-	+	+	-	-	-	+	+	23
ERS8	-	-	-	-	-	-	-	+	-	-	-	+	+	+	28
ERS10	-	-	-	-	-	-	-	+	+	-	-	-	+	+	23
ERS11	-	-	-	-	-	-	+	-	-	-	-	-	-	-	22
ERS12	-	-	-	-	-	-	-	+	+	-	-	-	+	+	23
ERS13	-	-	-	-	-	-	-	+	+	-	-	-	+	+	23
ERS14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
ERS15	-	-	-	-	-	-	-	-	-	-	-	+	+	+	28

Strain No:	Antiserum against strain no:- RDV 1	RDV 28	RDV 45	RDV 62	ERS 3a	ERS 18	ERS 20	ERS 22	ERS 54	ERS 31	ERS 37	ERS 40	ERS 46	ERS 47b	ERS 53
ERS16	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
ERS17	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
ERS18	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
ERS19	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
ERS20	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
ERS21	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
ERS22	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
ERS23	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+
ERS24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
ERS25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
ERS26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ERS27	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
ERS28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ERS29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ERS30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

Strain No:	Antiserum against strain no:- EQ2	EQ3	EQ7	NCTC 3365	NCTC 8529	NCTC 8644	NCTC 8987	ERS 12	EQ4	RDV 9	RDV 64	RDV 44	RDV 55	ERS 29	O group
ERS16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	14
ERS17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	12
ERS18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6
ERS19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6
ERS20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7
ERS21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	8
ERS22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	8
ERS23	-	-	-	-	+	-	-	-	-	-	-	-	-	-	20
ERS24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15
ERS25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15
ERS26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	17
ERS27	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15
ERS28	-	-	-	-	-	-	-	-	-	-	-	+	+	+	27
ERS29	-	-	-	-	-	-	-	+	-	-	-	+	+	+	28
ERS30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15

Strain No:	Antiserum against strain no:- RDV 1	RDV 28	RDV 45	RDV 62	ERS 3a	ERS 18	ERS 20	ERS 22	ERS 54	ERS 31	ERS 37	ERS 40	ERS 46	ERS 47b	ERS 53
ERS31	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
ERS32	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
ERS33	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ERS34	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ERS35	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
ERS36	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
ERS37	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
ERS38	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ERS39	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
ERS40	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
ERS41	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
ERS42	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
ERS43	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
ERS44	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
ERS45	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-

	Antiserum against strain no:-														
Strain No:	EQ2	EQ3	EQ7	NCTC 3365	NCTC 8529	NCTC 8644	NCTC 8987	ERS 12	EQ4	RDV 9	RDV 64	RDV 44	RDV 55	ERS 29	O group
ERS31	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10
ERS32	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10
ERS33	-	-	-	-	+	-	-	-	-	-	-	-	-	-	20
ERS34	-	-	-	-	+	-	-	-	-	-	-	-	-	-	20
ERS35	-	-	-	-	-	-	-	-	-	-	-	-	-	-	13
ERS36	-	-	-	-	-	-	-	-	-	-	-	-	-	-	13
ERS37	-	-	-	-	-	-	-	-	-	-	-	-	-	-	11
ERS38	-	-	-	-	-	-	-	-	-	-	-	+	+	+	27
ERS39	-	-	-	-	-	-	-	-	-	-	-	-	-	-	12
ERS40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	12
ERS41	-	-	-	-	-	-	-	-	-	-	-	-	-	-	14
ERS42	-	-	-	-	-	-	-	-	-	-	-	-	-	-	13
ERS43	-	-	-	-	-	-	-	-	-	-	-	-	-	-	13
ERS44	-	-	-	-	-	-	-	-	-	-	-	-	-	-	14
ERS45	-	-	-	-	-	-	-	-	-	-	-	-	-	-	13

Strain No:	Antiserum against strain no:- RDV 1	RDV 28	RDV 45	RDV 62	ERS 3a	ERS 18	ERS 20	ERS 22	ERS 54	ERS 31	ERS 37	ERS 40	ERS 46	ERS 47b	ERS 53
ERS46	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
ERS47a	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
ERS47b	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
ERS48a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ERS48b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ERS49a	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
ERS49b	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
ERS51	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ERS52	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
ERS53	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
ERS54	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
ERS55	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
ERS56	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	Antiserum against strain no:-														
Strain No:	EQ2	EQ3	EQ7	NCTC 3365	NCTC 8529	NCTC 8644	NCTC 8987	ERS 12	EQ4	RDV 9	RDV 64	RDV 44	RDV 55	ERS 29	O group
ERS46	-	-	-	-	-	-	-	-	-	-	-	-	-	-	13
ERS47a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	14
ERS47b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	14
ERS48a	-	-	+	-	-	-	-	-	-	-	-	-	-	-	18
ERS48b	-	-	+	-	-	-	-	-	-	-	-	-	-	-	18
ERS49a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3
ERS49b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3
ERS51	-	-	-	-	-	+	-	-	-	-	-	-	-	-	21
ERS52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	14
ERS53	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15
ERS54	-	-	-	-	-	-	-	-	-	-	-	-	-	-	9
ERS55	-	-	-	-	-	-	-	+	+	+	-	+	+	+	-
ERS56	+	-	-	-	-	-	-	-	-	-	-	-	-	-	16

Strain No:	Antiserum against strain no:- RDV 1	RDV 28	RDV 45	RDV 62	ERS 3a	ERS 18	ERS 20	ERS 22	ERS 54	ERS 31	ERS 37	ERS 40	ERS 46	ERS 47b	ERS 53
EQ1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EQ2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EQ3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EQ4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EQ5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
EQ6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EQ7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EQ8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
EQ9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EQ10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EQ11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EQ12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EQ13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EQ14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Strain No:	Antiserum against strain no:- EQ2	EQ3	EQ7	NCTC 3365	NCTC 8529	NCTC 8644	NCTC 8987	ERS 12	EQ4	RDV 9	RDV 64	RDV 44	RDV 55	ERS 29	O group
EQ1	-	+	-	-	-	-	-	-	-	-	-	-	-	-	17
EQ2	+	-	-	-	-	-	-	-	-	-	-	-	-	-	16
EQ3	-	+	-	-	-	-	-	-	-	-	-	-	-	-	17
EQ4	-	-	-	-	-	-	-	-	+	-	-	-	-	-	24
EQ5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15
EQ6	-	+	-	-	-	-	-	-	-	-	-	-	-	-	17
EQ7	-	-	+	-	-	-	-	-	-	-	-	-	-	-	18
EQ8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15
EQ9	-	-	-	-	-	-	-	-	-	-	-	-	+	+	28
EQ10	-	-	-	-	-	-	-	-	-	-	-	-	+	+	28
EQ11	-	-	-	-	-	-	-	-	-	-	-	-	+	+	28
EQ12	-	-	-	-	-	-	-	-	-	-	-	-	+	+	28
EQ13	-	-	-	-	-	-	-	-	-	-	-	-	+	+	28
EQ14	-	-	-	-	-	-	-	-	-	-	-	+	+	+	27

	Antiserum against strain no:-														
Strain No:	RDV 1	RDV 28	RDV 45	RDV 62	ERS 3a	ERS 18	ERS 20	ERS 22	ERS 54	ERS 31	ERS 37	ERS 40	ERS 46	ERS 47b	ERS 53
NCTC3365	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NCTC8529	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NCTC8644	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NCTC8794	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NCTC8987	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NCTC9435	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	Antiserum against strain no:-														
Strain No:	EQ2	EQ3	EQ7	NCTC 3365	NCTC 8529	NCTC 8644	NCTC 8987	ERS 12	EQ4	RDV 9	RDV 64	RDV 44	RDV 55	ERS 29	O group
NCTC3365	-	-	-	+	-	-	-	-	-	-	-	-	-	-	19
NCTC8529	-	-	-	-	+	-	-	-	-	-	-	-	-	-	20
NCTC8644	-	-	-	-	-	+	-	-	-	-	-	-	-	-	21
NCTC8794	-	-	-	-	-	+	-	-	-	-	-	-	-	-	21
NCTC8987	-	-	-	-	-	-	+	-	-	-	-	-	-	-	22
NCTC9435	-	-	-	-	-	+	-	-	-	-	-	-	-	-	21

APPENDIX C: Results of tube agglutination reactions between 138 strains of *Actinobacillus equuli* and 29 antisera prepared against heated bacterial suspensions

Figures indicate the reciprocal titre with strain indicated.

O = no agglutination at a dilution of 1 in 20.

nt = not tested.

Strain No:	Antiserum prepared against strain:- RDV 1	RDV 28	RDV 45	RDV 62	ERS 3a	ERS 18	ERS 20	ERS 22	ERS 54	ERS 31	ERS 37	ERS 40	ERS 46	ERS 47b	ERS 53
RDV1	320	0	0	0	0	0	0	0	20	0	20	0	20	0	20
RDV2	320	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RDV3	320	0	0	0	0	0	0	0	0	0	20	20	20	0	20
RDV4	0	20	0	0	20	40	20	20	20	0	80	20	0	0	20
RDV5	320	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RDV6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RDV7	0	0	0	0	0	0	0	0	0	0	0	0	20	0	0
RDV8	0	0	0	0	0	0	0	0	0	0	80	0	20	0	0
RDV9	0	0	0	0	0	0	0	0	0	0	0	0	20	40	0
RDV10	0	0	0	0	0	0	0	0	0	0	20	0	0	20	0
RDV11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RDV12	0	0	0	0	20	20	20	20	0	0	0	20	20	0	20
RDV13	0	20	0	0	0	0	20	0	0	0	0	20	20	0	20
RDV14	0	20	0	0	0	0	20	40	0	0	40	80	40	20	20
RDV15	0	0	0	0	nt	nt	0	0	nt	nt	0	0	0	0	0

Antiserum prepared against strain:-

Strain No:	EQ2	EQ3	EQ7	NCTC 3365	NCTC 8529	NCTC 8644	NCTC 8987	ERS 12	EQ4	RDV 9	RDV 64	RDV 44	RDV 55	ERS 29	O group
RDV1	0	0	0	0	0	0	0	0	0	0	0	0	20	20	1
RDV2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
RDV3	0	0	0	0	0	0	0	0	0	0	0	20	0	0	1
RDV4	20	0	0	0	0	20	0	20	80	20	320	40	0	0	26
RDV5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
RDV6	0	0	320	0	0	0	0	0	0	0	0	0	0	0	18
RDV7	0	0	0	0	0	20	0	20	0	0	0	40	640	2560	28
RDV8	0	0	0	0	0	20	0	20	0	20	40	40	320	1280	28
RDV9	0	0	0	0	0	0	0	20	80	640	40	0	20	20	25
RDV10	0	0	0	0	0	0	0	20	20	640	20	40	40	80	25
RDV11	160	0	0	0	0	0	0	0	0	0	0	20	0	0	16
RDV12	0	0	0	0	0	40	20	20	40	40	40	40	640	1280	28
RDV13	20	0	0	0	0	40	20	40	40	40	20	80	1280	2560	28
RDV14	20	0	0	0	0	0	0	1280	80	20	80	0	40	40	23
RDV15	160	0	0	0	0	0	0	0	nt	0	0	0	0	0	16

Antiserum prepared against strain:-

Strain No:	RDV 1	RDV 28	RDV 45	RDV 62	ERS 3a	ERS 18	ERS 20	ERS 22	ERS 54	ERS 31	ERS 37	ERS 40	ERS 46	ERS 47b	ERS 53
RDV16	0	0	0	0	0	0	0	0	0	0	40	0	20	0	20
RDV17	0	0	0	0	0	0	0	0	0	0	40	0	20	0	20
RDV18	0	0	0	0	0	0	0	0	0	0	40	0	20	0	20
RDV19	0	0	0	0	0	0	0	0	0	0	80	0	20	0	20
RDV20	0	0	0	0	0	0	0	0	0	0	0	0	0	320	0
RDV21	0	0	0	0	0	0	0	0	0	0	0	0	320	0	0
RDV22	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RDV23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RDV24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RDV25	0	0	0	0	0	0	0	0	0	0	20	0	0	0	0
RDV26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RDV27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RDV28	0	640	40	0	0	0	0	0	0	0	20	0	0	0	0
RDV29	0	640	20	0	nt	nt	0	0	nt	nt	0	0	0	0	0
RDV30	0	640	20	0	nt	nt	0	0	nt	nt	20	0	0	0	0

Strain No.	Antiserum prepared against strain:- EQ2	EQ3	EQ7	NCTC 3365	NCTC 8529	NCTC 8644	NCTC 8987	ERS 12	EQ4	RDV 9	RDV 64	RDV 44	RDV 55	ERS 29	O group
RDV16	20	0	0	0	0	20	0	20	40	0	20	80	160	1280	28
RDV17	20	0	0	0	0	20	0	20	40	0	0	80	160	1280	28
RDV18	40	0	0	0	0	20	0	40	40	0	0	80	320	1280	28
RDV19	20	0	0	0	0	20	0	40	40	0	0	80	320	1280	28
RDV20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14
RDV21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	13
RDV22	0	0	0	0	0	0	0	640	80	40	0	20	20	40	23
RDV23	0	0	0	0	0	0	0	640	80	80	0	0	20	40	23
RDV24	0	0	0	0	0	0	0	640	80	80	0	0	20	80	23
RDV25	0	0	0	0	0	0	0	640	80	40	0	20	20	80	23
RDV26	0	0	0	0	0	0	0	640	80	40	0	0	20	80	23
RDV27	0	0	0	0	0	0	0	640	80	40	0	20	20	80	23
RDV28	40	0	0	0	0	20	0	0	0	0	0	0	0	0	2
RDV29	20	0	0	0	0	0	0	0	nt	0	0	0	0	0	2
RDV30	40	0	0	0	0	20	0	0	nt	0	0	0	0	0	2

Antiserum prepared against strain:-

Strain No:	RDV 1	RDV 28	RDV 45	RDV 62	ERS 3a	ERS 18	ERS 20	ERS 22	ERS 54	ERS 31	ERS 37	ERS 40	ERS 46	ERS 47b	ERS 53
RDV31	0	640	40	0	nt	nt	0	0	nt	nt	0	0	0	0	0
RDV32	0	640	20	0	20	20	0	0	0	0	0	0	0	0	0
RDV33	0	0	20	0	40	40	20	0	40	20	160	0	20	0	20
RDV34	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RDV35	0	0	0	0	0	0	0	20	0	20	20	20	0	0	0
RDV36	0	0	0	0	0	0	0	20	0	0	40	20	0	0	0
RDV37	0	0	0	0	20	20	0	20	40	40	20	20	0	0	0
RDV38	0	20	20	0	20	20	20	0	40	20	40	0	40	0	20
RDV39	0	20	40	0	20	20	20	0	20	0	40	0	40	0	20
RDV40	0	20	40	0	0	0	40	0	0	0	40	0	40	0	40
RDV41	0	20	40	0	0	0	20	0	0	0	40	0	40	0	40
RDV42	0	0	0	0	0	0	0	0	0	0	20	0	0	0	20
RDV43	0	0	0	0	0	0	0	0	0	0	20	0	0	0	20
RDV44	0	0	0	0	0	0	0	0	0	20	20	0	0	0	0
RDV45	0	0	640	0	0	0	0	0	0	0	20	0	20	0	0

	Antiserum prepared against strain:-														
Strain No:	EQ2	EQ3	EQ7	NCTC 3365	NCTC 8529	NCTC 8644	NCTC 8987	ERS 12	EQ4	RDV 9	RDV 64	RDV 44	RDV 55	ERS 29	O group
RDV31	20	0	0	0	0	0	0	0	nt	0	0	0	0	0	2
RDV32	20	0	0	0	0	0	0	0	0	0	0	0	0	0	2
RDV33	20	0	0	0	0	20	0	20	40	20	0	0	40	40	11
RDV34	0	0	0	0	640	0	0	0	0	0	0	0	0	0	20
RDV35	0	0	0	0	0	0	0	0	40	0	20	640	40	160	27
RDV36	0	0	0	0	0	0	0	0	40	0	0	640	40	320	27
RDV37	0	0	0	0	0	0	0	0	80	0	0	640	40	320	27
RDV38	20	0	0	0	0	20	0	80	80	0	20	40	320	1280	28
RDV39	40	0	0	0	0	20	0	80	80	20	20	80	640	2560	28
RDV40	40	20	20	0	0	20	0	80	80	20	20	40	320	1280	28
RDV41	40	0	0	0	0	20	0	80	80	20	20	40	320	1280	28
RDV42	20	0	0	0	0	0	0	0	40	20	20	320	40	80	27
RDV43	20	0	0	0	0	0	0	0	40	0	0	320	80	160	27
RDV44	0	0	0	0	0	0	0	20	0	0	0	640	40	160	27
RDV45	20	0	0	0	0	20	0	0	0	20	0	0	0	0	3

Antiserum prepared against strain:-

Strain No:	RDV 1	RDV 28	RDV 45	RDV 62	ERS 3a	ERS 18	ERS 20	ERS 22	ERS 54	ERS 31	ERS 37	ERS 40	ERS 46	ERS 47b	ERS 53
RDV46	0	0	0	0	20	0	0	0	0	0	0	0	160	0	0
RDV50	0	20	0	0	0	0	20	20	0	0	20	20	20	0	20
RDV51	0	20	0	0	0	0	20	20	0	0	20	20	20	0	20
RDV52	0	0	0	0	20	0	0	160	20	0	0	0	0	0	0
RDV53	0	0	0	0	nt	nt	0	160	nt	nt	0	0	0	0	0
RDV54	0	0	0	0	20	20	0	20	0	0	40	0	20	0	20
RDV55	0	20	20	0	20	20	0	20	20	0	40	20	20	0	20
RDV56	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RDV59	40	0	0	0	0	0	0	40	0	0	40	20	0	20	20
RDV60	0	0	0	0	20	0	0	20	0	0	20	0	0	0	0
RDV61	0	0	0	320	0	0	0	0	0	0	0	0	0	0	0
RDV62	0	0	0	640	0	0	0	0	0	0	0	0	0	0	0
RDV63	0	0	0	320	0	0	0	0	0	0	0	0	0	0	0
RDV64	0	0	0	0	0	0	0	0	0	0	0	20	0	0	0
RDV65	0	0	0	0	0	0	0	0	0	0	0	20	0	0	0

	Antiserum prepared against strain:-														
Strain No:	EQ2	EQ3	EQ7	NCTC 3365	NCTC 8529	NCTC 8644	NCTC 8987	ERS 12	EQ4	RDV 9	RDV 64	RDV 44	RDV 55	ERS 29	O group
RDV46	0	0	0	0	0	0	0	0	0	0	0	0	0	0	13
RDV50	20	0	0	0	0	20	0	80	40	40	40	40	320	1280	28
RDV51	20	0	0	0	0	20	0	80	40	40	20	40	320	1280	28
RDV52	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8
RDV53	0	0	0	0	0	0	0	0	nt	0	0	0	0	0	8
RDV54	20	0	0	0	0	20	0	40	80	40	40	80	640	2560	28
RDV55	20	0	0	0	0	20	0	80	80	20	20	40	640	2560	28
RDV56	0	0	0	0	0	0	0	0	0	0	0	640	40	40	27
RDV59	20	0	0	0	0	20	0	640	80	0	20	20	20	80	23
RDV60	0	0	0	0	0	160	0	0	0	0	0	0	0	0	21
RDV61	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
RDV62	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
RDV63	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
RDV64	20	0	0	0	0	0	40	0	40	80	640	0	20	20	26
RDV65	20	0	0	0	0	0	0	40	20	40	320	20	20	80	26

Antiserum prepared against strain:-

Strain No:	RDV 1	RDV 28	RDV 45	RDV 62	ERS 3a	ERS 18	ERS 20	ERS 22	ERS 54	ERS 31	ERS 37	ERS 40	ERS 46	ERS 47b	ERS 53
ERS1	320	0	0	0	20	0	0	0	0	0	0	0	0	20	0
ERS2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ERS3a	0	0	0	0	640	0	0	0	40	0	0	0	0	0	0
ERS3b	0	0	0	0	640	0	0	0	20	0	0	0	0	0	0
ERS4	0	20	0	0	20	20	40	0	80	40	320	40	20	0	40
ERS5	0	0	0	0	0	0	0	20	0	0	320	20	20	0	40
ERS6	0	20	0	0	0	0	20	0	0	0	20	0	20	0	0
ERS7	0	0	0	0	0	0	0	0	0	0	40	0	20	0	0
ERS8	20	20	20	0	0	0	0	0	0	0	20	0	40	20	0
ERS10	0	20	0	0	20	0	0	0	0	0	80	20	20	0	20
ERS11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ERS12	0	20	0	0	0	0	0	0	0	0	40	0	40	0	0
ERS13	0	0	0	0	20	0	0	20	0	0	40	0	0	0	20
ERS14	320	0	0	0	0	0	0	0	0	0	0	0	20	0	0
ERS15	0	0	0	0	0	0	20	20	0	0	20	0	20	20	0

	Antiserum prepared against strain:-														
Strain No:	EQ2	EQ3	EQ7	NCTC 3365	NCTC 8529	NCTC 8644	NCTC 8987	ERS 12	EQ4	RDV 9	RDV 64	RDV 44	RDV 55	ERS 29	O group
ERS1	0	0	0	0	0	0	0	0	0	0	0	20	0	0	1
ERS2	0	0	0	0	0	40	0	0	0	0	0	0	0	0	
ERS3a	0	0	0	0	0	0	0	0	20	0	0	0	0	0	5
ERS3b	0	0	0	0	0	0	0	0	20	0	0	0	0	0	5
ERS4	40	0	0	40	0	40	40	0	40	20	40	0	40	0	11
ERS5	0	0	0	0	0	40	0	0	0	0	0	0	0	0	11
ERS6	20	0	0	0	0	20	0	20	20	0	20	40	640	1280	28
ERS7	0	0	0	0	0	0	0	640	80	20	20	40	20	160	23
ERS8	40	20	0	0	0	80	0	40	80	20	0	40	640	2560	28
ERS10	0	0	0	0	0	0	0	640	160	40	40	20	0	80	23
ERS11	0	0	0	0	0	0	320	0	0	0	0	0	0	0	22
ERS12	0	40	0	0	0	20	20	1280	160	20	20	20	80	80	23
ERS13	20	0	0	0	0	20	0	640	160	20	20	0	0	160	23
ERS14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
ERS15	0	0	0	0	0	20	0	20	80	40	20	40	640	2560	28

Strain No:	Antiserum prepared against strain:- RDV 1	RDV 28	RDV 45	RDV 62	ERS 3a	ERS 18	ERS 20	ERS 22	ERS 54	ERS 31	ERS 37	ERS 40	ERS 46	ERS 47b	ERS 53
ERS16	0	0	0	0	0	0	0	0	0	0	0	0	0	320	0
ERS17	0	0	0	0	0	0	0	0	0	0	0	640	0	0	0
ERS18	0	0	0	0	0	640	0	0	0	0	0	0	0	80	40
ERS19	0	0	0	0	0	320	0	0	0	0	0	0	0	0	0
ERS20	0	0	20	0	0	0	320	0	0	0	80	0	20	0	0
ERS21	0	0	0	0	0	0	0	320	0	0	0	0	0	20	0
ERS22	0	0	0	0	nt	nt	0	320	nt	nt	0	0	0	20	0
ERS23	0	0	0	0	0	0	0	0	0	0	0	0	20	0	40
ERS24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	320
ERS25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	320
ERS26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ERS27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	320
ERS28	0	20	0	0	0	0	20	20	0	0	40	20	0	0	40
ERS29	0	40	40	0	0	0	0	0	0	0	40	0	20	0	20
ERS30	0	0	0	0	0	0	0	0	40	20	0	0	0	0	320

Strain No:	Antiserum prepared against strain:- EQ2	EQ3	EQ7	NCTC 3365	NCTC 8529	NCTC 8644	NCTC 8987	ERS 12	EQ4	RDV 9	RDV 64	RDV 44	RDV 55	ERS 29	O group
ERS16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14
ERS17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12
ERS18	0	0	0	0	0	0	0	0	0	20	0	0	0	0	6
ERS19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6
ERS20	20	0	0	0	0	0	0	0	0	0	0	0	0	0	7
ERS21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8
ERS22	0	0	0	0	0	0	0	0	nt	0	0	0	0	0	8
ERS23	0	0	0	0	320	0	0	0	0	0	0	0	0	0	20
ERS24	0	0	0	0	0	0	0	0	20	0	0	0	0	0	15
ERS25	0	0	0	0	0	0	0	0	40	0	0	0	0	0	15
ERS26	0	80	0	0	0	0	0	0	0	0	0	0	0	0	17
ERS27	0	0	0	0	0	0	0	0	40	0	0	0	0	0	15
ERS28	20	0	0	0	0	80	80	0	0	20	40	320	40	80	27
ERS29	40	0	0	0	0	40	40	160	80	20	20	160	640	2560	28
ERS30	0	0	0	0	0	0	0	0	80	0	0	0	0	0	15

Strain No:	Antiserum prepared against strain:- RDV 1	RDV 28	RDV 45	RDV 62	ERS 3a	ERS 18	ERS 20	ERS 22	ERS 54	ERS 31	ERS 37	ERS 40	ERS 46	ERS 47b	ERS 53
ERS31	0	0	0	0	0	0	0	0	20	640	0	0	0	0	0
ERS32	0	0	0	0	0	0	0	0	0	640	0	0	0	0	0
ERS33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ERS34	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ERS35	0	0	0	0	0	0	0	0	0	0	0	0	320	0	0
ERS36	0	0	0	0	0	0	0	0	0	0	0	0	160	0	0
ERS37	0	0	0	0	0	20	0	0	0	0	320	0	0	0	0
ERS38	0	0	0	0	0	0	0	0	0	0	20	0	0	0	20
ERS39	0	0	0	0	0	0	0	0	0	20	0	640	0	0	0
ERS40	0	0	0	0	0	20	0	0	0	40	0	640	40	0	0
ERS41	0	0	0	0	0	0	0	0	0	0	0	0	0	320	0
ERS42	0	0	0	0	0	0	0	0	0	0	0	0	320	0	0
ERS43	0	0	0	0	20	20	0	0	20	40	0	0	160	40	0
ERS44	0	0	0	0	nt	nt	0	0	nt	nt	0	0	0	640	0
ERS45	0	0	0	0	20	0	0	0	20	0	0	0	160	0	0

	Antiserum prepared against strain:-														
Strain No:	EQ2	EQ3	EQ7	NCTC 3365	NCTC 8529	NCTC 8644	NCTC 8987	ERS 12	EQ4	RDV 9	RDV 64	RDV 44	RDV 55	ERS 29	O group
ERS31	0	0	0	0	0	0	0	0	20	0	0	0	0	0	10
ERS32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10
ERS33	0	0	0	0	640	0	0	0	0	0	0	0	0	0	20
ERS34	0	0	0	0	640	0	0	0	0	0	0	0	0	0	20
ERS35	0	0	0	0	0	0	0	0	0	0	0	0	0	0	13
ERS36	0	0	0	0	0	0	0	0	0	0	0	0	0	0	13
ERS37	20	0	0	0	0	0	0	0	0	0	0	0	0	0	11
ERS38	20	0	0	0	0	20	0	0	20	20	20	320	40	160	27
ERS39	0	0	0	0	0	0	0	0	20	0	0	0	0	0	12
ERS40	40	0	0	0	0	20	20	0	40	20	0	0	0	0	12
ERS41	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14
ERS42	0	0	0	0	0	0	0	0	0	0	0	0	0	0	13
ERS43	0	0	0	0	0	0	0	0	0	20	0	0	0	0	13
ERS44	0	0	0	0	0	0	0	0	nt	0	0	0	0	0	14
ERS45	0	0	0	0	0	0	0	0	0	0	0	0	0	0	13

Antiserum prepared against strain:-

Strain No:	RDV 1	RDV 28	RDV 45	RDV 62	ERS 3a	ERS 18	ERS 20	ERS 22	ERS 54	ERS 31	ERS 37	ERS 40	ERS 46	ERS 47b	ERS 53
ERS46	0	0	0	0	0	0	0	0	0	0	0	0	320	0	0
ERS47a	0	0	0	0	nt	nt	0	0	nt	nt	0	0	0	320	0
ERS47b	0	0	0	0	0	0	0	0	0	0	0	0	0	640	0
ERS48a	0	0	0	0	nt	nt	0	0	nt	nt	20	0	0	0	0
ERS48b	0	0	0	0	20	20	20	20	0	0	80	20	20	0	20
ERS49a	0	0	320	0	nt	nt	0	0	nt	nt	0	0	0	0	0
ERS49b	0	0	320	0	0	0	0	0	0	20	0	0	0	0	0
ERS51	0	0	0	0	0	0	0	20	0	0	40	20	0	20	0
ERS52	0	0	0	0	20	0	0	0	0	0	0	0	0	640	0
ERS53	0	0	0	0	20	0	0	20	0	0	0	0	0	0	640
ERS54	0	0	0	0	40	0	0	160	640	0	0	0	0	0	0
ERS55	0	20	20	0	0	0	20	20	0	0	80	0	0	20	40
ERS56	0	40	0	0	20	20	20	20	40	20	40	0	0	20	0

Strain No:	Antiserum prepared against strain:- EQ2	EQ3	EQ7	NCTC 3365	NCTC 8529	NCTC 8644	NCTC 8987	ERS 12	EQ4	RDV 9	RDV 64	RDV 44	RDV 55	ERS 29	O group
ERS46	0	0	0	0	0	0	0	0	0	20	0	0	0	0	13
ERS47a	0	0	0	0	0	0	0	0	nt	0	0	0	0	0	14
ERS47b	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14
ERS48a	0	0	320	0	0	0	0	0	nt	0	0	0	0	0	18
ERS48b	0	0	160	0	0	0	0	0	20	0	20	0	0	0	18
ERS49a	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
ERS49b	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
ERS51	0	0	0	0	0	160	0	0	20	0	20	0	0	0	21
ERS52	0	0	0	0	0	0	0	0	80	20	20	0	0	0	14
ERS53	20	0	0	0	0	0	0	0	0	20	0	0	0	0	15
ERS54	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9
ERS55	20	0	0	0	0	40	0	80	160	80	40	80	40	160	
ERS56	160	0	0	0	0	0	0	0	40	20	20	40	0	0	16

Antiserum prepared against strain:-

Strain No:	RDV 1	RDV 28	RDV 45	RDV 62	ERS 3a	ERS 18	ERS 20	ERS 22	ERS 54	ERS 31	ERS 37	ERS 40	ERS 46	ERS 47b	ERS 53
EQ1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
EQ2	0	0	0	0	0	0	0	0	20	0	0	0	0	0	0
EQ3	0	0	0	0	0	0	0	0	0	0	20	0	0	0	0
EQ4	0	0	0	0	20	20	0	40	20	20	0	40	0	0	0
EQ5	0	0	0	0	nt	nt	0	0	nt	nt	0	0	0	0	160
EQ6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
EQ7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
EQ8	0	0	0	0	nt	nt	0	0	nt	nt	0	0	0	0	160
EQ9	0	0	0	20	nt	nt	0	20	nt	nt	0	0	40	20	20
EQ10	80	0	0	20	nt	nt	40	20	nt	nt	20	0	80	20	20
EQ11	80	0	20	20	nt	nt	40	20	nt	nt	40	0	80	40	40
EQ12	40	0	20	20	nt	nt	20	20	nt	nt	40	0	40	20	20
EQ13	40	20	40	0	nt	nt	20	20	nt	nt	40	0	80	20	20
EQ14	0	0	0	0	0	0	0	20	0	20	40	20	0	0	0

Strain No:	Antiserum prepared against strain:- EQ2	EQ3	EQ7	NCTC 3365	NCTC 8529	NCTC 8644	NCTC 8987	ERS 12	EQ4	RDV 9	RDV 64	RDV 44	RDV 55	ERS 29	O group
EQ1	0	80	0	0	0	0	0	0	0	0	0	0	0	0	17
EQ2	160	0	0	20	0	0	0	0	40	20	0	0	0	0	16
EQ3	0	160	0	0	0	0	0	0	20	0	0	0	0	0	17
EQ4	20	0	0	0	0	0	0	320	640	0	0	20	40	160	24
EQ5	0	0	0	0	0	0	0	0	nt	0	0	0	0	0	15
EQ6	0	160	0	0	0	0	0	0	0	0	0	0	0	0	17
EQ7	0	0	640	0	0	0	0	0	0	0	0	0	0	0	18
EQ8	0	0	0	0	0	0	0	0	nt	0	0	0	0	20	15
EQ9	0	0	0	0	0	0	0	20	nt	20	20	20	1280	2560	28
EQ10	20	0	0	0	0	40	20	20	nt	80	80	20	1280	2560	28
EQ11	20	0	0	20	20	40	20	40	nt	80	80	20	1280	2560	28
EQ12	0	20	20	0	0	20	0	20	nt	40	20	20	1280	2560	28
EQ13	20	0	0	20	20	40	0	80	nt	40	40	20	1280	2560	28
EQ14	0	0	0	0	0	20	0	0	0	0	20	640	40	160	27

Antiserum prepared against strain:-

Strain No:	RDV 1	RDV 28	RDV 45	RDV 62	ERS 3a	ERS 18	ERS 20	ERS 22	ERS 54	ERS 31	ERS 37	ERS 40	ERS 46	ERS 47b	ERS 53
NCTC3365	0	20	40	0	0	20	20	0	20	0	20	0	20	0	20
NCTC8529	0	0	0	0	0	0	0	0	20	0	0	0	0	0	0
NCTC8644	20	0	0	0	0	0	0	0	0	0	20	0	0	0	0
NCTC8794	0	0	0	0	20	0	0	0	0	0	0	0	0	0	0
NCTC8987	0	0	0	0	0	0	0	0	0	0	20	0	0	0	0
NCTC9435	0	0	0	0	40	0	0	0	0	0	0	0	0	0	0

	Antiserum prepared against strain:-														
Strain No:	EQ2	EQ3	EQ7	NCTC 3365	NCTC 8529	NCTC 8644	NCTC 8987	ERS 12	EQ4	RDV 9	RDV 64	RDV 44	RDV 55	ERS 29	O group
NCTC3365	40	0	0	640	0	20	40	20	40	40	80	0	20	20	19
NCTC8529	0	0	0	0	640	0	0	0	20	0	0	0	0	0	20
NCTC8644	0	0	0	0	0	1280	0	0	0	0	0	0	0	0	21
NCTC8794	0	0	0	0	0	160	0	0	0	0	0	20	0	0	21
NCTC8987	0	0	0	0	0	0	320	0	0	0	0	0	0	0	22
NCTC9435	0	0	0	0	0	1280	0	0	0	0	0	0	0	0	21

APPENDIX D: Results of immunodiffusion precipitin tests between 138 strains of *Actinobacillus equuli* and 10 antisera prepared against heated bacterial suspensions

Figures indicate number of the precipitin line(s) developed after overnight incubation at room temperature.

Reactions obtained with homologous system are indicated by underlined figures.

O indicates no visible reaction.

> indicates the overlapping of precipitin lines.

Antiserum prepared against strain:-

Strain No:	RDV 1	RDV 28	ERS 22	ERS 37	ERS 53	NCTC 3365	NCTC 8529	NCTC 8644	ERS 12	ERS 29
RDV1	3	0	0	0	0	1	0	0	0	0
RDV2	3	0	0	0	0	1	0	0	0	0
RDV3	3	0	0	0	0	1	0	0	0	0
RDV4	0	0	0	0	0	1	0	0	0	1
RDV5	3	0	0	0	0	1	0	0	0	0
RDV6	0	0	0	0	0	1	0	0	0	0
RDV7	0	0	0	0	0	1	0	1	1	3
RDV8	0	0	0	0	0	1	0	0	0	2
RDV9	0	0	0	0	0	1	0	0	0	0
RDV10	0	0	0	0	0	1	0	0	0	1
RDV11	0	0	0	0	0	1	0	0	0	0
RDV12	0	0	0	0	0	1	0	0	1	2
RDV13	0	0	0	0	0	1	0	0	2	3
RDV14	0	0	0	0	0	1	0	1	3	2
RDV15	0	0	0	0	0	1	0	0	0	0
RDV16	0	0	0	0	0	1	0	1	1	2
RDV17	0	0	0	0	0	1	0	1	1	2
RDV18	0	0	0	0	0	1	0	1	1	2
RDV19	0	0	0	0	0	1	0	1	2	3
RDV20	1	0	0	0	0	1	0	0	0	0
RDV21	0	0	0	0	0	1	0	0	0	0
RDV22	0	0	0	0	0	1	0	0	2	0
RDV23	0	0	0	0	0	1	0	0	2	0
RDV24	0	0	0	0	0	1	0	0	2	1
RDV25	0	0	0	0	0	1	0	0	2	1

Antiserum prepared against strain:-

Strain No:	RDV 1	RDV 28	ERS 22	ERS 37	ERS 53	NCTC 3365	NCTC 8529	NCTC 8644	ERS 12	ERS 29
RDV26	0	0	0	0	0	1	0	0	2	1
RDV27	0	0	0	0	0	1	0	0	2	1
RDV28	0	2	0	0	0	1	0	0	0	0
RDV29	0	2	0	0	0	1	0	0	0	0
RDV30	0	2	0	0	0	1	0	0	0	0
RDV31	0	2	0	0	0	1	0	0	0	0
RDV32	0	2	0	0	0	1	0	0	0	0
RDV33	0	0	0	>1	0	1	0	0	0	0
RDV34	0	0	0	0	0	1	2	0	0	0
RDV35	0	0	0	0	0	1	0	0	1	2
RDV36	0	0	0	0	0	1	0	1	1	2
RDV37	0	0	0	0	0	1	0	0	1	2
RDV38	1	0	0	0	0	1	0	1	1	2
RDV39	1	0	0	0	0	1	0	1	1	2
RDV40	1	0	0	0	0	1	0	1	1	2
RDV41	1	0	0	0	0	1	0	1	1	2
RDV42	0	0	0	0	0	1	0	0	1	2
RDV43	0	0	0	0	0	1	0	0	1	2
RDV44	0	0	0	0	0	1	0	0	1	2
RDV45	0	0	0	0	0	1	0	0	0	0
RDV46	0	0	0	0	0	1	0	0	0	0
RDV50	0	0	0	0	0	1	0	0	0	2
RDV51	0	0	0	0	0	1	0	0	0	2
RDV52	0	0	0	0	0	1	0	0	0	0
RDV53	0	0	0	0	0	1	0	0	0	0

Antiserum prepared against strain:-

Strain No:	RDV 1	RDV 28	ERS 22	ERS 37	ERS 53	NCTC 3365	NCTC 8529	NCTC 8644	ERS 12	ERS 29
RDV54	0	0	0	0	0	1	0	0	1	2
RDV55	0	0	0	0	0	1	0	0	1	2
RDV56	0	0	0	0	0	1	0	0	1	1
RDV59	0	0	1	0	0	1	0	1	1	2
RDV60	0	0	0	0	0	1	0	2	0	0
RDV61	0	0	0	0	0	1	0	0	0	0
RDV62	0	0	0	0	0	1	0	0	0	0
RDV63	0	0	0	0	0	1	0	0	0	0
RDV64	0	0	0	0	0	1	0	0	0	1
RDV65	0	0	0	0	0	1	0	0	0	1
ERS1	>1	0	0	0	0	1	0	0	0	0
ERS2	0	0	0	0	0	1	0	0	0	0
ERS3a	0	0	0	0	0	1	0	1	0	0
ERS3b	0	0	0	0	0	1	0	1	0	0
ERS4	0	0	0	>1	0	1	0	0	0	0
ERS5	0	0	0	>1	0	1	0	0	0	0
ERS6	0	0	0	0	0	1	0	0	1	2
ERS7	0	0	0	0	0	1	0	0	2	1
ERS8	0	0	0	0	0	1	0	1	2	3
ERS10	0	0	0	0	0	1	0	0	2	1
ERS11	0	0	0	0	0	1	0	0	0	0
ERS12	0	0	0	0	0	1	0	0	2	1
ERS13	0	0	0	0	0	1	0	0	2	1
ERS14	>2	0	0	0	0	1	0	0	0	0
ERS15	0	0	0	0	0	1	0	0	1	2

Antiserum prepared against strain:-

Strain No:	RDV 1	RDV 28	ERS 22	ERS 37	ERS 53	NCTC 3365	NCTC 8529	NCTC 8644	ERS 12	ERS 29
ERS16	0	0	0	0	0	1	0	0	0	0
ERS17	0	0	0	0	0	1	0	0	0	0
ERS18	0	0	0	0	0	1	0	0	0	0
ERS19	0	0	0	0	0	1	0	0	0	0
ERS20	0	0	0	0	0	1	0	0	0	0
ERS21	0	0	2	0	0	1	0	1	0	0
ERS22	0	0	2	0	0	1	0	1	0	0
ERS23	0	0	0	0	0	1	2	0	0	0
ERS24	0	0	0	0	2	1	0	0	0	0
ERS25	0	0	0	0	2	1	0	0	0	0
ERS26	0	0	0	0	0	1	0	0	0	0
ERS27	0	0	0	0	2	1	0	0	0	0
ERS28	0	0	0	0	0	1	0	0	2	3
ERS29	0	0	0	0	0	1	0	1	2	3
ERS30	0	0	0	0	2	1	0	0	0	0
ERS31	0	0	0	0	0	1	0	0	0	0
ERS32	0	0	0	0	0	1	0	0	0	0
ERS33	0	0	0	0	0	1	2	0	0	0
ERS34	0	0	0	0	0	1	2	0	0	0
ERS35	0	0	0	0	0	1	0	0	0	0
ERS36	0	0	1	0	0	1	0	1	0	0
ERS37	0	0	0	2	0	1	0	0	0	0
ERS38	0	0	0	0	0	1	0	0	2	2
ERS39	0	0	0	0	0	1	0	0	0	0
ERS40	0	0	0	0	0	1	0	0	0	0

Antiserum prepared against strain:-

Strain No:	RDV 1	RDV 28	ERS 22	ERS 37	ERS 53	NCTC 3365	NCTC 8529	NCTC 8644	ERS 12	ERS 29
ERS41	0	0	0	0	0	1	0	0	0	0
ERS42	0	0	0	0	0	1	0	1	0	0
ERS43	0	0	0	0	0	1	0	1	0	0
ERS44	0	0	0	0	0	1	1	1	0	0
ERS45	0	0	0	0	0	1	0	0	0	0
ERS46	0	0	0	0	0	1	0	0	0	0
ERS47a	0	0	0	0	0	1	1	1	0	0
ERS47b	0	0	0	0	0	1	1	1	0	0
ERS48a	0	0	0	0	0	1	0	0	0	0
ERS48b	0	0	0	0	0	1	0	0	0	0
ERS49a	0	0	0	0	0	1	0	0	0	0
ERS49b	0	0	0	0	0	1	0	0	0	0
ERS51	0	0	0	0	0	1	0	2	0	0
ERS52	0	0	0	0	0	1	0	0	0	0
ERS53	0	0	0	0	>1	1	0	0	0	0
ERS54	0	0	0	0	0	1	0	0	0	0
ERS55	0	0	0	0	0	1	0	0	1	1
ERS56	0	0	0	0	0	1	0	0	0	0
EQ1	0	0	0	0	0	1	0	1	1	1
EQ2	1	0	0	0	0	1	0	1	1	0
EQ3	0	0	0	0	0	1	0	1	1	1
EQ4	0	0	0	0	1	1	0	0	1	1
EQ5	0	0	0	0	2	1	0	1	1	1
EQ6	0	0	0	0	0	1	0	1	1	1
EQ7	0	0	0	0	0	1	0	0	0	0

Antiserum prepared against strain:-

Strain No:	RDV 1	RDV 28	ERS 22	ERS 37	ERS 53	NCTC 3365	NCTC 8529	NCTC 8644	ERS 12	ERS 29
EQ8	0	0	0	0	0	1	0	1	1	1
EQ9	0	0	0	0	0	1	0	0	0	2
EQ10	0	0	0	0	0	1	0	0	0	>1
EQ11	0	0	0	0	0	1	0	0	0	2
EQ12	0	0	0	0	0	1	0	0	0	>1
EQ13	0	0	0	0	0	1	0	1	1	3
EQ14	0	0	0	0	0	1	0	0	1	2
NCTC3365	0	0	0	0	0	2	0	0	0	0
NCTC8529	0	0	0	0	0	1	2	0	0	0
NCTC8644	0	0	0	0	0	1	0	>2	1	0
NCTC8794	0	0	0	0	0	1	0	2	0	0
NCTC8987	0	0	0	0	0	1	0	1	1	1
NCTC9435	0	0	0	0	0	1	0	>2	1	0

APPENDIX E: Biochemical and fermentative properties of strains of Actinobacillus suis and Actinobacillus lignieresi investigated in the present study.

All the organisms included in this appendix gave the reactions listed below with the following tests and substrates :-

Catalase	+	Ammonia	-	Citrate	-
Gelatin	-	Gluconate	-	Indole	-
MacConkey	+	Malonate	-	M.R.	-
Nitrate	+	ONPG	+	Phenylalanine	-
Phosphatase	+	Urease	+		
Rhamnose	-	Xylose	+	Dextrose	+
Fructose	+	Maltose	+	Sucrose	+
Inulin	-	Adonitol	-	Sorbitol	-
Dulcitol	-	Inositol	-		

Key:

Biochemical tests:

+ = positive reaction.
± = weak positive reaction.
(+)= late positive reaction.
- = negative reaction.
α = partial haemolysis
β = complete haemolysis

Fermentative activities:

+ = fermentation with acid but no gas within 24 hours.
+2, +5 = fermentation after 2, 5 days.
- = no fermentation within 14 days.

Test for	A. suis strains									
	NCTC10841	NCTC10843	1688/72	27585/72	927/73	928/73	15296/73	18808/73	18809/73	32452/73
Oxidase	(+)	+	(+)	+	(+)	-	-	(+)	(+)	+
Aesculin	+	+	+	+	+	+	+	+	+	+
Hippurate	+	+	+	+	+	+	+	+	+	+
H_2S	-	-	-	-	+	-	-	-	-	-
KCN	-	-	-	-	-	-	-	-	-	-
Methylene blue	±	-	-	±	+	-	+	-	-	±
Glucose iod.	-	-	-	-	-	-	-	-	-	-
Maltose iod.	-	-	-	-	-	-	-	-	-	-
V.P. reaction	-	-	-	-	-	-	-	-	-	-
Haemolysis(SBA)	β	β	β	β	β	β	β	β	β	β
Haemolysis(HBA)	α	α	α	α	α	α	α	α	α	α
Arabinose	+	+	+	+	+	+	+2	+3	+2	+
Galactose	+2	+3	+2	+2	+	+	+3	+3	+	+
Mannose	+	+3	+	+2	+	+	+2	+	+3	+3
Cellobiose	+2	+	+	+	+	+2	+2	+2	+3	+2
Lactose	+	+	+	+	+	+	+	+	+	+
Melibiose	+	+	+	+2	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+	+	+	+
Raffinose	+	+	+	+	+	+	+	+	+	+
Dextrin	+	+	+	+2	+	+	+	+	+	+
Starch	-	-	-	+14	-	-	-	+10	-	-
Glycerol	+5	+4	+4	+6	+4	+4	+5	+3	+5	+4
Mannitol	-	-	-	-	-	-	-	-	-	-
Salicin	+	+	+	+	+	+	+	+	+	+

Test for	A. suis strains						A. lignieresi strains		
	3665 9/73	37558/73	4015/74	P247	P380	P393	A2	A3	A5
Oxidase	-	+	-	-	-	-	(+)	(+)	+
Aesculin	+	+	+	+	+	+	-	-	-
Hippurate	+	+	+	+	-	+	-	-	-
H_2S	-	-	-	-	-	-	+	+	+
KCN	-	-	-	-	-	-	-	-	-
Methylene blue	+	-	±	-	-	±	+	+	+
Glucose iod.	-	-	-	-	-	-	±	-	±
Maltose iod.	±	-	±	-	-	±	+	+	+
V.P. reaction	-	-	-	-	-	-	-	-	+
Haemolysis(SBA)	β	β	β	β	β	β	-	-	-
Haemolysis(HBA)	β	β	α	α	α	α	-	-	-
Arabinose	+	+	+	+	+	+	-	-	-
Galactose	+	+	+	+	+	+	+	+	+
Mannose	+3	+3	+2	+	+	+	+	+	+
Cellobiose	+	+2	+	+	+	+2	-	-	-
Lactose	+	+	+	+	+	+	+2	+2	+2
Melibiose	+	+	+	+2	+	+	-	-	-
Trehalose	+	+	+	+	+	+	-	-	-
Raffinose	+	+	+	+	+	+	-	-	-
Dextrin	+	+	+	+	+	+2	+3	+	+2
Starch	-	-	-	-	-	-	-	-	-
Glycerol	+3	+5	+3	+3	+3	+3	+5	+3	+3
Mannitol	-	-	-	-	-	-	+	+	+
Salicin	+	+	+	+	+	+	-	-	-

Test for	A. lignieresi strains									
	A6	A7	A13	A14	A17	A20	A43	A49	A53	A100
Oxidase	(+)	(+)	(+)	(+)	(+)	+	+	+	+	-
Aesculin	-	-	-	-	-	-	-	-	-	-
Hippurate	-	-	-	-	-	-	-	-	-	-
H_2S	+	+	+	+	+	+	+	+	+	+
KCN	-	-	-	-	-	-	-	+	-	+
Methylene blue	+	+	+	+	+	+	+	+	+	+
Glucose iod.	±	+	±	-	-	-	-	-	+	±
Maltose iod.	+	-	+	±	+	-	+	+	+	±
V.P. reaction	+	-	-	-	-	-	-	-	-	-
Haemolysis(SBA)	-	-	-	-	-	-	-	-	-	-
Haemolysis(HBA)	-	-	-	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-	-	-	-
Galactose	+	+	+	+2	+2	+	+	+2	+3	+
Mannose	+	+	+	+	+	+	+	+2	+	+
Cellobiose	-	-	-	-	-	-	-	-	-	-
Lactose	+3	+3	+3	+5	+2	+3	+2	+2	+3	+2
Melibiose	-	-	-	-	-	-	-	-	-	-
Trehalose	-	-	-	-	-	-	-	-	-	-
Raffinose	-	-	+3	-	-	-	+3	-	-	-
Dextrin	+	+6	+2	+2	+3	+	+	+2	+	+
Starch	-	-	-	-	-	-	-	-	-	-
Glycerol	+3	+3	+3	+2	+4	+4	+3	+5	+3	+3
Mannitol	+	+	+	+	+	+	+	+	+	+
Salicin	-	-	-	-	-	-	-	-	-	-