A COMPARISON OF THE GUINEA PIG INTRACUTANEOUS TEST, CHICK TEST, AND CHICK EMBRYO TEST, FOR USE IN DETECTING THE VIRULENCE OF THREE TYPES OF CORYNEBACTERIUM DIPHTHERIAE

by

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INTRODUCTION

Since the identification of Corynebacterium diphtheriae as the causative agent in diphtheria, laboratory methods have been sought which would accurately determine the virulence and toxigenic potential of any given culture. The methods used should have to be simple and not too expensive for the average laboratory. Since cultures isolated from active cases of the disease may be considered virulent and toxigenic, the cultures of greatest interest in this connection were those isolated from suspected carriers of the disease. Even though the use of improved methods of treatment and the development of efficient means of immunization, coupled with almost universal immunization of children, has for the most part placed this disease in the background, the problem of carrier checking is still of importance due to fluctuations in the occurrence of the disease.

Various tests have been proposed for the detection of virulence and toxigenicity of this organism. Included among the more prominent are the guinea pig subcutaneous test, the guinea pig intracutaneous test, the mouse intracerebral test, the rabbit skin test, and the chick test. In addition there is also the problem of culture identification. This is due in part to variations in culture reaction, and morphology. It has been suggested that certain of the standard tests for virulence and toxigenicity might be supplemented by more sen-

sitive tests. It was also suggested that these standard tests might be evaluated as to relative efficiency in detecting virulence and toxigenicity.

This work was undertaken with the following problem in mind: 1. To check several of the standard tests as to relative efficiency in detecting the virulence and toxigenicity of this organism, 2. To evaluate the relative sensitivity of chick embryos in detecting the virulence and toxigenicity of cultures in the light of results obtained by using the standard tests, 3. To determine the possibility of false positive virulence tests being encountered as a result of the virulence of closely related organisms.

The tests selected for comparison were the guinea pig intracutaneous test, the chick test, and the use of chick embryos. The intracutaneous test was selected since it was the test being used routinely in the laboratory where this research was undertaken. The chick test is of relatively recent origin. Therefore, the necessity of evaluating it in the light of the guinea pig test was considered necessary. The use of chick embryos as an indicator of virulence and toxigenicity was suggested by the works of Goodpasture, Cromartie, Evans, and Tsun Tung. The purpose of the test was to determine if the death of inoculated chick embryos, in the absence of specific antitoxin, is a satisfactory indication of the virulence and toxigenicity of the cultures under investigation.

The techniques used in the guinea pig intracutaneous test, the chick test, and the basis for identification were taken from those compiled in the book: Diagnostic Procedures and Reagents of The American Public Health Association (1941). The testing and identifications followed the procedures listed in this manual to produce results in line with those which might be encountered in most public health laboratories.

The control cultures used were known stock cultures of virulent and toxigenic strains from the stock culture collection of The Kansas Public Health Laboratories. The second group was made up of cultures isolated from specimens sent to the above mentioned laboratory for bacteriological analysis. All cultures which did not exhibit typical morphology when stained with alkaline methylene blue, and those cultures failing to stain gram positive were discarded. Approximately 500 cultures were submitted with 387 cultures being used throughout the entire work.

This research problem was undertaken and completed in the Division of Public Health Laboratories of The Kansas State Board of Health.

LITERATURE REVIEW

The bacteriology of diphtheria has presented two distinct problems. The first problem is the actual isolation and identification of the organism, and second, the detection and measurement of virulence. Kolmer (1912) attempted a summation of the facts known at that time with particular emphasis being placed on the use of morphological classifications, and biometric analysis. His conclusions were that the three morphological types which he encountered, granular, barred, and solid staining, were associated with varying degrees of severity in the disease. The granular types were shown to predominate in the larger portion of clinical cases, the solid staining type being found more frequently at the termination of the disease than at the beginning. The results of his biometric tests showed that the virulent diphtheria cultures produced acid more frequently from dextrose and dextrin, results obtained with other carbohydrates varying considerably. Kolmer and Moshage (1916) attempted to link the formation of acids in the fermentation of carbohydrates with the production of soluble toxins. To do this, virulent strains of the organism were grown in the presence of increasing amounts of antitoxin, the purpose of this being to neutralize the toxins as they were formed. When this was conducted in the presence of fermentable carbohydrates, it was found that no appreciable change in the fermentation ability of the cultures under

study was encountered. The conclusions drawn were that the carbohydrate fermentation of Corynebacterium diphtheriae was dependent upon the action of secondary enzymes, and that the action of these enzymes was independent of the production of extracellular soluble toxins. This work failed to show any relation between toxin formation and the fermentation of carbohydrates, but it did hint, in the light of Kolmer's previous work, the possibility that several sub-types of the organism might exist; these sub-types being distinct in their biometric reactions and morphology, and in their relative virulence also. However, to clarify the difference between virulence and toxin formation, Cary (1917), while studying the problem of virulence and toxin formation, found that virulence and toxigenicity are not inseparable. It was found by him that many strains which tested a virulent were strong toxin producers. Therefore, the problem of toxin formation became of prime importance irrespective of the relative virulence of the culture under examination. Much later Crowell (1926), using a single cell isolation strain, concluded that morphological types have no hereditary significance or relation to virulence and toxin formation. He also concluded that non-toxic strains are mutants of toxic forms, and that non-toxic forms are permanently non-toxic. These findings diverge from the earlier findings of Kolmer (1912) that morphological types are distinct, and that virulent forms fail in one morphological group while non-toxic

forms fall in another group.

When considering the use of carbohydrates to enhance the production of toxins, other factors of media must be considered such as reaction and the utilization of nutrients which are added in media to support the growth of Corynebacterium diphtheriae. Davis and Ferry (1919) reported on the elaboration of toxin in broth cultures. Their findings tended to refute the findings of Hadley (1907) and others who claimed the production of strong toxin in purely synthetic media. Broth containing only amino acids and inorganic salts was found incapable of supporting the growth of the diphtheria organism. They did find that the addition of 0.5 percent infusion bouillon would allow the organisms to grow and multiply. It was also found, however, that ten percent bouillon in their media was necessary for the elaboration of toxin. They concluded from these findings that the diphtheria organism did not synthesize a toxin, but that the toxin was a catabolic substance elaborated by the organism only in the presence of certain materials supplied by the bouillon. The use of bacteriological peptones in the production of toxins was investigated by Davis (1920). He found that the presence of peptones in media used for the production of toxin greatly enhanced toxin formation. Wilcox (1922) investigated the use of bacteriological peptones and went a step further when he came to the conclusion that different peptone preparations varied in their ability to

enhance the elaboration of toxin. Wadsworth and Wheeler (1928) published work on the problem of attenuation and toxin production in synthetic media. They encountered only one culture of the organism which would grow in synthetic media. This growth was found to be accompanied by marked attenuation and change in morphology, culture characteristics, carbohydrate fermentations, and immunological reactions with complete loss of virulence and toxigenicity. This seemed to confirm the work of Davis and Ferry with synthetic media. It was found, however, that a medium composed of chlorides, sulfates, and phosphates of sodium, calcium, and magnesium, with dextrose as a source of energy and peptone as a source of nitrogen, was sufficient for the production of toxin. Maver (1930) investigated synthetic media with similar results being encountered, indicating the uncertain status of synthetic media in the production of toxins. Further clarification was attempted by Mueller, Klise, Porter, and Greybiel (1933). It was concluded that good growth of the organism and production of toxin is obtained on synthetic media containing proteose peptone. Proteose peptone was found to contain creatine which is found in animal tissue, suggesting the presence of tissue extracts. Therefore, the proteose peptone was assumed to be similar in composition to infusions. It was also shown that tryptophane is one of the essential factors necessary for the growth of the various strains of this organism.

With the separation of the traits of virulence and toxigenicity, means were sought to use cultural characteristics as indications of virulence and toxigenicity, with the main emphasis being placed on the production of toxins. Heeren and Magrail (1930), investigating the subject, worked on the relation of hemolysin to the production of toxins and the virulence of the organism. They found this characteristic unreliable, as an indication of either toxigenicity or virulence, and also transient, generally disappearing with continued cultivation of the culture being studied. They found that the ability to produce hemolysin might be regained by passing the culture through a series of animal inoculations. In the same year Heeren (1930) found that the formation of pellicles seems to accompany the ability to cause disease and give positive virulence tests. This was shown by the fact that 89 out of 100 toxigenic cultures produced pellicles. That the formation of a pellicle is not necessary for the formation of toxins was shown by the fact that the remaining 11 of the cultures were toxigenic strains of known virulence. A possible reason for these results may be found in the work of Anderson, Happold, McLeod, and Thompson (1931) who, working together, reported on the existence of two distinct subtypes of Corynebacterium diphtheriae, and a third type which was found intermediate in its reactions. The three sub-types reported by these workers are: Corynebacterium diphtheriae gravis, Corynebacterium diphtheriae mitis, and the third,

Corynebacterium diphtheriae intermedius. This work was elaborated on further by McLeod (1932), Anderson, Cooper, Happold, and McLeod (1933). They attributed the more severe cases of diphtheria to Corynebacterium diphtheriae gravis, the less severe to Corynebacterium diphtheriae mitis; Corynebacterium diphtheriae intermedius was found to vary between these two types in virulence. This classification was based on the biometric reactions of the organism.

Besides attempts to relate hemolysin and pellicle formation to toxin formation, other ways have been sought with which to relate biometric reactions to the formation of toxin and to the virulence of the organism. Further work on the use of dextrose as a material to enhance the production of toxin formation was undertaken by Hagen and Heller (1932). They found that dextrose added twice daily in a concentration of 0.15 percent increased the toxin titre of the base, providing the medium was alkalinized at frequent intervals. Maltose was found to act similarly without the pH adjustment required in the case of dextrose. Their conclusions were that dextrose in small amounts is superior to maltose, dextrin, and glycerol. The final pH was found not to be an infallible index as to the best time for the toxin harvest.

Povitzki, Eisner, and Jackson (1933) confirmed the relationship between the three types of organisms described by McLeod and the men working with him. They found toxins formed by the three types were identical, and that antitoxins

formed by the injection of toxins from the three were identical. The difference between the three in disease production was therefore believed to be one of degree and not of kind.

The Animal Tests

The first animal test to be considered was the guinea pig intracutaneous test. Weston and Kolmer (1911) set down their basis for the use of guinea pigs in testing for the virulence of Corynebacterium diphtheriae. Their object was to achieve the most favorable set of conditions for the production of toxins, and for their use in animals possessing no marked degree of immunity or resistance to the toxin. They advocated the use of alkaline broth media containing some carbohydrate, preferably dextrose, in a concentration of 1.00 percent. They suggested that all cultures should be incubated at 37° C. for at least 48 hours. They also proposed a crude test using a suspension of diphtheria organisms washed from a 48 hour Loeffler slant, using normal saline as the suspending fluid. Later Kolmer, Woody, and Moshage (1916) published results obtained with the guinea pig test. They elaborated further on their techniques, advocating the use of dextrose broth as a suspending fluid for the organism, in animal inoculations. Kolmer and Moshage (1916) published further work on the problem of virulence testing, giving an

evaluation of the various methods used at that time to test for virulence. In this work they set down their basis for an accurate virulence test. The factors as listed are given below:

1. The test must be as delicate as possible in order that it may reveal the potential harmfulness of bacilli of low virulence.

2. Any evidence of virulence, however slight, must be regarded as a positive indication of the pathogenicity of the culture.

3. The test must be conducted with pure cultures.

4. The test must be conducted with as large a dose of the culture under study as possible.

Results of their experiments show that the injection of the same culture subcutaneously, and intracutaneously vary slightly in the final result, the intracutaneous route being inferior to the subcutaneous injection. This is offset in part by the fact that more than one culture can be tested on a single animal if the inoculations are made intracutaneously. They found that cultures suspended in saline yielded results similar to those obtained with broth cultures and suspensions washed from slants with broth. This varies from Kolmer's earlier work from which he advocated the use of broth as a suspending fluid. Later work by Stark, Sherman, and Stark (1928) emphasized the necessity of using pure cultures as had Kolmer and his associates. It was found that the toxin of diphtheria was readily destroyed by bacteria often encountered in field cultures. The use of saline as a suspending fluid was not confirmed, however, Holt and Wright (1940) found

saline to be inferior to broth, thus agreeing with Kolmer's earlier findings. They found that suspending the organisms in saline or distilled water caused a loss of virulence, and generally death of the cultures in a very short time.

The chick test was based on the work of Frobisher (1940). His work dealt with the susceptibility of chicks to the action of the diphtheria bacilli and diphtheria toxin. Later, Frobisher, Parsons, and Tsun Tung (1942) developed this work into the chick test. The chicks were injected with one cubic centimeter of a 48 hour broth culture of the organism. These tests were run in parallel with the rabbit skin test. It was found that both tests gave similar results. The gravis cultures were found ti kill the chicks before the mitis cultures but the difference was not considered to be significant.

The use of chick embryos as a culture media for viruses is not new. The technique of opening incubated chicken eggs for the study of embryos in such a manner as to insure survival of the embryo has been investigated by many. Byerling (1926) gave a simple technique for opening the shell in a work on suffocation of chick embryos. He managed to keep them alive to the fifth and eighth days. Others found that embryos can be cultivated in open shell to hatching if the shell is opened after the sixth day. After the sixth day embryos in the open shell were marked by a greater resistance and adaptability to environment. Goodpasture (1932) studied the vaccinal infection of the chick embryo and re-

ported the work as showing the possibility of using embryos in the cultivation of the virus. This was confirmed later by Buddingh (1936) who published work on the generalized vaccinal infection of chick embryos. He recommended the use of chick embryos which had been incubated for at least 12 days. Goodpasture (1937) enlarged the field of interest by writing on the problem of infection by bacterial invasion of the chorio-allantoic membrane of chick embryos. He found most pathogens able to develop in the embryonic cells; exceptions were S. aureus, St. hemolyticus, and Corynebacterium diphtheriae. He employed two strains of diphtheria in this experiment, the first from a case of acute mitral endocarditis, the second being the standard Park strain VIII. All strains killed the embryos within 48 hours. Flaky colonies were observed on the chorio-allantoic membrane with surrounding blood vessels exhibiting signs of hemorrhage and necrosis. Controls set up by the addition of antitoxin to the inoculated embryos survived. Evans (1938) in studying the effects of diphtheria toxin on the chick embryo confirmed the work of Goodpasture on the susceptibility of the embryos to diphtheria toxin. The embryos were found to be more susceptible to the action of diphtheria toxin on the basis of weight than were guinea pigs. Instead of recommending 12-day chick embryos as had Buddingh (1936), chick embryos incubated for ten days were recommended. Cromartie (1941), using the technique of Goodpasture, confirmed these previous findings of the action of

Corynebacterium diphtheriae by the injection of cultures into the egg in such a manner as to render the naso-pharynx available for infection. Clumps of bacilli were found in the nasal passages and ulcerative lesions were found to be numerous. His conclusions were that the developing chick embryo is susceptible to infection with Corynebacterium diphtheriae with the mouth and naso-pharynx serving as a portal of entry. Tsun Tung (1945) examined the problem further, using cultures of virulent and avirulent organisms. He found that cultures injected through the chorio-allantoic route all produced growth but that the avirulent strains failed to kill the embryos within 48 hours. This further confirmed the findings of Goodpasture and others and brought out the fact that those cultures which are avirulent and not toxin formers would not kill the embryos within a 48 hour period.

Basis of Identification

Following the identification system of McLeod and others (1931, 1932, 1933), the cultures were grouped according to their ability to ferment dextrose, starch, and sucrose. Other biometric reactions used were the formation of hemolysins, production of pellicles on liquid media, and their ability to revert media to an alkaline reaction. The ability of some of the cultures to liquify Loeffler's medium was also noted since this is a trait common to Corynebacterium pyogenes, a closely

related animal pathogen, Ward (1917), Brown and Ocult (1930), and Lowell (1939).

The characteristics as listed for the three types of Corynebacterium diphtheriae by McLeod and others are as follows:

Corynebacterium diphtheriae gravis

1. Appearance on tellurite media--forty-eight hour colonies grey to grey black.

2. Morphology--Short diphtheroid usually without granules. On Loeffler's the granules are well marked but may be scanty.

3. Pellicle--Pellicles are formed on liquid media with distinct granulation of the culture throughout the media.

4. Reversion--Broth cultures may revert to an alkaline condition.

5. Hemolysins--The production of hemolysins is usually negative but is considered variable.

6. Fermentation of carbohydrates--Acid is formed in dextrose, galactose, and maltose. No acid is formed in sucrose. Invariably this type ferments starch.

Corynebacterium diphtheriae mitis

1. Tellurite medium--Forty-eight hour colonies are black.

2. Morphology--Varies considerably, usually long forms with granules.

3. Pellicle formation--Usually late, with usually uniform turbidity of the fluid, or mixed uniform and granular turbidity.

4. Reversion--This trait is absent.

5. Hemolysins--Hemolysins are invariably produced.

6. Fermentation of carbohydrates--Acid is formed in dextrose, galactose, and maltose. No acid is formed in sucrose or in starch.

Corynebacterium diphtheriae intermedius

1. Appearance on tellurite medium--Forty-eight hour colonies are grey.

2. Morphology--Varies considerably.

3. Pellicle formation--Usually negative.

4. Reversion--This trait is absent.

5. Hemolysins--Hemolysins are not produced.

6. Fermentation of carbohydrates--This organism produces acid in dextrose, galactose, and maltose, but fails to ferment sucrose or starch.

The main characteristics listed in Bergey's Manual of Determinative Bacteriology (1939) for Corynebacterium pyogenes are:

1. Blood serum--Coagulated slants pitted by digestion, usually followed by complete destruction of the medium.

2. Hemolysins--The organism produces hemolysins.

3. Reversion--Reversion of media to alkaline condition is common.

4. Fermentation of carbohydrates--Acid is formed in dextrose, sucrose, lactose, xylose, and starch but not from raffinose, inulin, mannitol or salicin. The intravenous injection of rabbits with this organism is fatal.

The composition of the media used, and the technique used in detecting hemolysins is given in the Appendix. All media used were checked against standard cultures. The fermentation tests were incubated for five days at 37° C. The tests were read at 48 hours and at the end of a five day period. All biometric reactions were run in duplicate to check on the reliability of the results.

TECHNIQUE OF THE VIRULENCE TESTS

The Chick Test

This test was conducted using the technique of Frobisher, Parsons, and Tsun Tung (1942). The chick was purchased at the age of 24 hours and was held for seven days before inoculation to insure the use of healthy stock in the testing. All chicks were from pullorum-tested flocks.

The cultures to be used were incubated at 37° C. for 48 hours. The chicks were labeled by the insertion of numbered wing bands in the web of the right wing, the left wing being reserved for inoculations. The chicks were divided into two equal groups, one group receiving from 40 to 60 units of diphtheria antitoxin. After an hour had elapsed the inoculations were begun. Each bird was injected with one cubic centimeter of a broth culture. The injection was made into the dorsal aerolar connective tissue of the left wing. The injection was accomplished by using a sterile two milliliter syringe to which was attached a sterile 26 gauge needle. A separate syringe and needle were used with each individual culture. The chicks were then observed over a period of 72 hours. Usually the strongly positive cultures killed the test chicks within 24 hours. However, some cultures required the full 72 hours to kill the birds. The general symptoms observed were found to be an early paralysis of the wing

injected followed by a more or less general paralysis of the bird. The birds would stagger, fall, and fail to regain their footing. When this occurred, death usually followed quite rapidly. Sterile broth was injected in some to act as a control on any toxic factors which might be present in the broth before inoculation with cultures took place.

The Guinea Pig Intracutaneous Virulence Test

This test consisted of the injection of a suspension of the culture under study intracutaneously in the guinea pig. Each pig was carefully shaved on the back, sides, and belly. Each side from the ridge of the backbone to the median line of the belly was marked off in five squares approximately four square centimeters in size, thus allowing for the injection of ten cultures on the back of one animal. Each pig was then ear tagged. The tags were put in the pig's ear several days before the inoculation to insure the retention of the tag. Two pigs were used with each group of ten cultures, one serving as a test animal and the other as a control on the test animal. Each control animal received 200 units of antitoxin about three hours before the injection of the cultures took place. The antitoxin was administered intraperitoneally.

The cultures used were grown on Loeffler slants for 48 hours. To each culture was added three-quarters of a milliliter of sterile toxin broth. The growth was then suspended

in this broth and drawn into a two millliliter syringe equipped with a sterile 26 gauge needle which was used to administer the injection. Then 0.1 millliliter of this suspension was injected intracutaneously on the designated square on the control and on the test guinea pig. A separate sterile syringe and needle were used with each culture under investigation. Diagramatic sketches were made of the back of each guinea pig, with the position of each culture being noted, and the ear tag number of each pig being noted also. The tests were read at the end of 24 hours, and at the end of 48 hours. A positive test was indicated by the formation of a spot of necrosis surrounded by an inflamed area. As a control on the broth, 0.1 millliliter of sterile broth was injected in each pig.

The Chick Embryo Test

The eggs used in this test were obtained from local hatcheries. The eggs were either incubated in the laboratory or incubated by the hatchery for the ten day period. These eggs were carefully candled before inoculation to insure the use of healthy embryos. The shell was carefully cleaned and then a small area of the shell was ground from the underlying membrane. The eggs were then divided into three groups; one group receiving 40 units of antitoxin, this group to act as a control. The three groups were then inocu-

lated with a suspension of organisms washed from a 48 hour Loeffler slant with sterile toxin broth. The injection was accomplished by use of a sterile two milliliter syringe to which was attached a 26 gauge needle. A separate sterile needle and syringe were used with each individual culture. The scraped area was then covered with Scotch tape and the number of the culture was marked on the shell. The eggs were then returned to the incubator. At the end of 24 hours one test egg from each group of three was examined. The eggs were opened by cutting off the shell over the air sack. The underlying membrane was then removed with forceps and the embryo examined. This process was repeated at the end of the 48 hour period with the control being opened also. Death of the embryo within the 48 hour period, the control culture surviving was recorded as a positive test. When examined, clumps of bacteria were observed on the membranes along with areas of necrosis and hemorrhage. Negative embryos and control embryos on being opened revealed undamaged membranes, the embryos showing movement when stimulated.

CULTURE HANDLING AND RESULTS

The cultures as they were received were streaked on McLeod's tellurite medium to isolate the diphtheria organisms. At the end of 48 hours suspicious colonies were picked from the McLeod's medium and transferred to Loeffler's medium. They were also examined by staining with methylene blue and

by gram staining technique at this time. Those cultures which consisted of typical organisms were saved. These cultures were classified according to their biometric reactions; their fermentation of dextrose, sucrose, and starch; their ability to revert media to an alkaline reaction; and the production of hemolysins and pellicles. These cultures were then inoculated into the three test animals.

The results obtained are compiled in the accompanying tables. Table 1 is a summary of the types of organisms found, Table 2 is a compilation of the results obtained with the three virulence tests and their relation to each other in the light of the three types; gravis, mitis, and intermedius. Table 3 gives the reactions of the control cultures, Table 4 gives a summary of virulence reactions of Corynebacterium pyogenes and Table 5 shows the relation of pellicle formation and hemolysin to the three types of diphtheria.

DISCUSSION

When the cultures were separated on the basis of biometric reaction, and the Corynebacterium diphtheriae cultures were segregated according to type, it was found that 14.21 percent of the cultures were Corynebacterium diphtheriae gravis, 4.69 percent of the cultures were found to be Corynebacterium diphtheriae mitis, and 29.91 percent of the cultures were found to be Corynebacterium diphtheriae intermedius. Of the remaining cultures, 22.22 percent were found to be

Table 1. A summary of the reactions and virulence tests of each variety and the number of each encountered.*

No. of each found	Dextrose	Sucrose	Starch	Hemolysin	Proteolysis	Pellicle	Reversion	Chick test	Chick control	Pig test	Pig control	Embryo 24	Embryo 48	Embryo control	Variety of organism
9	+	-	-	-	-	-	-	+	-	+	-	+	+	-	Corynebacterium diphtheriae intermedius
21	+	-	-	-	-	-	-	+	-	-	-	+	+	-	Corynebacterium diphtheriae intermedius
30	+	-	-	-	-	-	-	-	-	-	-	+	+	-	Corynebacterium diphtheriae intermedius
53	+	-	-	-	-	-	-	-	-	-	-	-	-	-	Corynebacterium diphtheriae intermedius
5	+	-	-	+	-	±	-	+	-	+	-	+	+	-	Corynebacterium diphtheriae mitis
2	+	-	-	+	-	±	-	+	-	-	-	+	+	-	Corynebacterium diphtheriae mitis
7	+	-	-	+	-	±	-	-	-	-	-	+	+	-	Corynebacterium diphtheriae mitis
5	+	-	-	+	-	±	-	-	-	-	-	-	-	-	Corynebacterium diphtheriae mitis
12	+	-	+	±	-	+	+	+	-	+	-	+	+	-	Corynebacterium diphtheriae gravis
18	+	-	+	±	-	+	+	+	-	-	-	+	+	-	Corynebacterium diphtheriae gravis

Table 1 (concl.).

No. of each found	Dextrose	Sucrose	Starch	Hemolysin	Proteolysis	Pellicle	Reversion	Chick test	Chick control	Pig test	Pig control	Embryo 24	Embryo 48	Embryo control	Variety of organism
19	+	-	+	±	-	+	+	-	-	-	-	+	+	-	Corynebacterium diphtheriae gravis
6	+	-	+	±	-	+	+	-	-	-	-	-	-	-	Corynebacterium diphtheriae gravis
34	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Corynebacterium pyogenes
4	+	+	+	+	+	+	+	+	+	-	-	+	+	+	Corynebacterium pyogenes
5	+	+	+	+	+	+	+	+	+	-	-	-	-	-	Corynebacterium pyogenes
17	+	+	+	+	+	+	+	+	+	+	+	-	-	-	Corynebacterium pyogenes
22	+	+	+	+	+	+	+	-	-	-	-	-	-	-	Corynebacterium pyogenes
6	+	+	+	+	+	+	+	-	-	-	-	+	+	-	Corynebacterium pyogenes
387	(This total number includes those organisms found not to be diphtheria and non-virulent).														

* This summary does not include those organisms which were not diphtheria and which were not virulent.

Table 2. Percentage distribution of the three strains of diphtheria according to the total number of cultures examined, and according to their reaction with each of the test animals.

Percentage of each strain found virulent	Percentage of strain found non-virulent	Each strain's percentage of true diphtheria	Percentage virulent in all three tests	Percentage virulent for embryos only	Percentage virulent for embryos and chicks only	Percentage virulent in chicks	Percentage virulent in pigs	Percentage virulent in embryos	Name of strain
89.09	10.91	39.98	24.49	37.71	30.71	61.22	24.90	100.00	Corynebacterium diphtheriae gravis
73.68	26.32	11.38	33.57	50.00	37.77	50.00	33.57	100.00	Corynebacterium diphtheriae mitis
53.09	46.91	48.78	15.00	50.00	35.00	50.00	15.00	100.00	Corynebacterium diphtheriae intermedius

Table 3. A Summary of the reactions and virulence test of the five control cultures.

Dextrose	Sucrose	Starch	Hemolysins	Pellicles	Reversion	Chick test	Chick control	Pig test	Pig control	Embryo 24	Embryo 48	Embryo control	Culture designation	Variety of organism
+	-	-	-	-	-	+	-	+	-	+	+	-	Toronto	Corynebacterium diphtheriae intermedius
+	-	+	+	+	+	+	-	+	-	+	+	-	VII	Corynebacterium diphtheriae gravis
+	-	+	+	+	+	+	-	-	-	+	+	-	XIEM	Corynebacterium diphtheriae gravis
+	-	+	+	+	+	+	-	+	-	+	+	-	VIIEM	Corynebacterium diphtheriae gravis
+	-	+	+	+	+	+	-	+	-	+	+	-	C. dip. (Foltz)	Corynebacterium diphtheriae gravis

Table 4. The virulence reactions of the Corynebacterium pyogenes found among the cultures studied.

Percentage of pyogenes found to be virulent " " " " "	74.41	53.12	9.37	25.65	6.25
Percentage of pyogenes found virulent in all three tests " " " " "					
Percentage found virulent for embryos only " " " " "					
Percentage found virulent for chicks only " " " " "					
Percentage found virulent for pigs and chicks only " " " " "					
Percentage found virulent for chicks and embryos only " " " " "					
Percentage found negative for embryos only " " " " "					25.65
Percentage found negative for chicks only " " " " "					0
Percentage found negative for pigs only " " " " "					0
Percentage found virulent for chicks " " " " "					37.50
Percentage found virulent for pigs " " " " "					79.68
Percentage found virulent for embryos					68.75

Table 5. Relation of hemolysin and pellicle formation to virulence.

Percentage of virulent cultures producing hemolysins	Percentage of non-virulent cultures producing hemolysins	Percentage of virulent cultures producing pellicles	Percentage of non-virulent cultures producing pellicles	Strain
100.00	100.00	76.92	83.33	Corynebacterium diphtheriae mitis
0	0	0	0	Corynebacterium diphtheriae intermedius
53.06	50.00	100.00	100.00	Corynebacterium diphtheriae gravis

Corynebacterium pyogenes, and 28.97 percent to be non-virulent cultures of Corynebacterium which were not identified due to their lack of virulence. This proportional division of the cultures shows a rather large proportion of intermedius type with only a very small proportion of mitis being found present.

After the cultures were separated according to identity, it was found on the basis of the three virulence tests that 89.09 percent of the gravis, 73.66 percent of the mitis, and 53.09 percent of the intermedius were virulent for the test animals. The very high percentage of virulent mitis cultures present may be due to the very few mitis cultures encountered, being from active cases of the disease. The percentage given for the gravis parallels the figures given by McLeod and his associates (1931, 1932, 1933). The percentage of intermedius cultures encountered, and the percentage of virulent intermedius cultures are very high, and may indicate a trend in types and virulence of cultures generally encountered in the area from which these cultures were taken. This is borne out by the very small number of mitis cultures encountered. Usually the most numerous type encountered in this area is the mitis type. That there is not a greater difference between the percentage of virulent gravis and virulent intermedius cultures is probably due to the very large percentage of virulent gravis cultures.

With an evaluation of the results obtained with each test animal, according to culture type, it was found that the vir-

ulent gravis strains were virulent for 61.22 percent of the chicks, 24.49 percent of the guinea pigs, and 100.00 percent of the chick embryos. This shows a relatively low gravis virulence for the guinea pig, and the chick, in relation to the results obtained with the chick embryo. If the gravis strains are to be considered the more potent of the three, and the organism generally encountered in severe cases of the disease, as has been intimated by McLeod and his associates, these results may indicate one of two things. They may indicate a shift in gravis virulence, or they may indicate that the chick test and the guinea pig test are not picking up cultures which may be potentially dangerous. The results just mentioned may be borne out in the results obtained with the mitis and intermedius cultures. It was found that 50.00 percent of the virulent mitis were virulent for the chick, 33.57 percent of them for the guinea pig, and 100.00 percent of the virulent cultures were found virulent for the chick embryo. While the mitis cultures are not generally suspected of causing as many severe cases of the disease as are the gravis, the results obtained with this type appear to generally parallel the findings encountered with the gravis. The intermedius cultures act similarly. Of the intermedius, 50.00 percent were found to be virulent for the chick and 50.00 percent for the guinea pig. The results obtained with the guinea pig test are the same proportionally as those obtained with the chick test, indicating perhaps variations in

animal susceptibility to different strains. The embryos gave positive virulence tests with all cultures found virulent for the other two tests and 50.00 percent more in each case. This emphasizes the probable encounter of negative results with cultures which are virulent, when the guinea pig test and chick tests are used. The fact that these tests were run with identical cultures indicates a lack of sensitivity in the guinea pig and in the chick tests as now constituted.

Taking each of the strains as a separate entity, it was found that 24.49 percent of the virulent gravis cultures gave positive results in all three of the tests. Only 33.57 percent of the virulent mitis, and 15.00 percent of the virulent intermedius were found to be virulent for all three of the test animals. These percentages further emphasize the relative greater sensitivity of the chick embryo in detecting virulence. It was also found that 37.71 percent of the virulent gravis were virulent for embryos only. As to the others, 50.00 percent of the intermedius and 50.00 percent of the mitis were found to give similar results.

Taking each test separately, it was found that 61.22 percent of the gravis were virulent for the chicks, 24.90 percent of the gravis were virulent for the guinea pig, and that all virulent cultures were virulent for the chick embryo. Intermedius cultures were found less virulent for the chick, and less virulent for the guinea pig. Of the virulent intermedius cultures, 50.00 percent were found virulent for the chick and

15.00 percent virulent for the guinea pig. Taking the virulent mitis cultures as a whole, 50.00 percent were found virulent for the chick and 33.57 percent for the guinea pig. This indicates an increased mitis virulence for the guinea pig.

These percentages are relative and are based on the percentage of positives obtained with the chick embryo test as all indications of virulence in the guinea pig test and chick test were duplicated and surpassed by the chick embryo test.

Taking the Corynebacterium pyogenes as a separate unit, it was found that 74.41 percent of these cultures were virulent for the test animals, showing a high degree of virulence for the animals used in testing for virulence. Of the cultures of this organism, 53.12 percent were found virulent in all three tests, only 9.37 percent being found virulent for embryos only.

Previously mention has been made of the work of Heeren in determining the relation between pellicle formation and homolysin production in relation to virulence. Our results show the production of hemolysins by the mitis cultures, with variable results being encountered with the gravis cultures. The intermedius cultures were found not to produce hemolysins. This bears out the findings of Heeren that hemolysins production did not correlate with virulence. The inability of the intermedius, and a large proportion of the gravis, to produce hemolysins accounts for these results, as the intermedius and gravis cultures were found to make up the larger portion of the

virulent cultures encountered. These findings were also backed up by the findings of McLeod and his associates. The production of pellicles being common to all except the intermedius gives the reason for the correlation offered by Heeren between pellicle formation and virulence. However, many cultures which did not produce pellicles were found virulent. Heeren also noted this in his works.

CONCLUSIONS

Perhaps the main conclusion to be drawn from this work is the superiority of the chick embryo test in detecting the virulence of Corynebacterium diphtheriae. This was shown by the much greater percentage of positive virulence tests obtained with the chick embryos. This was further emphasized by the parallel results obtained when the reactions of each strain were examined. The fact that none of the test animals gave nearly the percentage results obtained with the chick embryos is significant. The nearest in sensitivity was the chick test, but even its best results fell far short of the results obtained with the chick embryo. It might be argued that the embryo test is too sensitive in detecting virulence, however, if the work of Kolmer and Moshage (1916) is followed, in which was stated that any indication of virulence, however slight, must be regarded as a positive test for virulence, the chick embryo test must be regarded as far superior to the guinea

pig and chick tests. The fact that specific antitoxin was able to protect the embryos for the 48 hour period of the test indicates that the positive results encountered were due to the action of toxins elaborated by the organism, and not due to bacterial invasion of the embryo. If the disease is to be considered one of toxin damage, the formation of toxins in the presence of the chick embryo, and its detection by means of the embryo's death, must be regarded as a positive indication of the organism's potential role in disease production.

It has been intimated from this work that the guinea pig virulence test, using intracutaneous inoculations, is not efficient as now constituted. The reason for this may be the fact that the cultures are injected in the skin in such small amounts that any toxins present will not be detected due to their inability to overcome the defenses of the guinea pig's skin. Kolmer and Mosage (1916) concluded in their requisites for a good virulence test that any indication of virulence, however slight, should be regarded as a positive test. This test as it is now constituted does not make provision for those reactions which can neither be called virulent or non-virulent. The possibility arises that many of the cultures which fall into this category will be called non-virulent even when they are virulent.

The chick test gave a much higher percentage of positive virulence tests than did the guinea pig test probably for several reasons. One reason was the fact that the organisms were injected in the broth in which they had been grown for a 48

hour period. By doing this, not only were organisms injected but also the toxins which they had elaborated during that time. The guinea pig test might have given similar results if the organisms had been administered in a similar manner. Another factor was the large inoculum employed in comparison to the amount administered to the guinea pig. However, Frobisher (1942) stated that the amount of inoculum employed did not apparently alter the reaction of time of death of the chick. The factors of reading were, of course, simplified in the chick test by using the death of the chick as the indication of virulence.

Other corynebacteria present, exclusive of the Corynebacterium pyogenes, were found to give negative virulence tests and therefore their presence is not considered of particular interest. The fact that both tests and controls gave positive virulence reactions with the Corynebacterium pyogenes indicates that there is probably little relation between any toxins which may be produced by this organism and Corynebacterium diphtheriae. The fact that the controls also died or gave positive virulence tests precludes the possibility of mistaking a positive virulence reaction with pyogenes for a positive virulence test with diphtheria organisms. The possibility might arise, however, in which an extremely toxic culture of Corynebacterium diphtheriae might overcome the antitoxin present and give results similar to the results obtained by the injection of Corynebacterium pyogenes.

SUMMARY

1. It was found that the guinea pig intracutaneous test for virulence is not as sensitive to the action of the three types of Corynebacterium diphtheriae as are the chick test and the chick embryo test.

2. The chick embryo test was found far more sensitive to the three types of Corynebacterium diphtheriae than are the guinea pig intracutaneous test and the chick test.

3. The fact that the chick embryos can be protected from the action of the toxin formed by Corynebacterium diphtheriae by the use of specific antitoxin would indicate that the chick embryos could be used for virulence tests.

4. It was also shown that the chick embryo test detects virulence which cannot be detected by the other two tests, thereby minimizing the possibility of getting negative reactions with virulent cultures.

5. It was shown that Corynebacterium pyogenes, an organism closely related to Corynebacterium diphtheriae, would give positive virulence tests with the animals used, but the fact that the controls receiving diphtheria antitoxin gave positive virulence tests also, minimizes the possibility of confusion when testing for the virulence of Corynebacterium diphtheriae.

6. It was also concluded that the correlations discov-

ered by Heeren between pellicle formation and virulence and the lack of correlations between hemolysin and virulence, could be explained on the basis of the work of McLeod and his associates.

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BIBLIOGRAPHY

American Public Health Association.
Diagnostic procedures and reagents of the American Public Health Association. Standard methods committee on diagnostic procedures and reagents. 1941.

Anderson, J. S., K. E. Cooper, F. C. Happold and J. W. McLeod.
Incidence and correlation with clinical severity of gravis, mitis, and intermedius diphtheria bacilli in a series of 500 cases in Leeds. Jour. Path. and Bact. 36: 169-182. 1933.

Anderson, J. S., F. C. Happold, J. W. McLeod and J. G. Thompson. On the existence of two forms of diphtheria bacilli, B. diphtheriae gravis, and B. diphtheriae mitis: And a new medium for the differentiation and for the bacteriological diagnosis of diphtheria. Jour. Path. and Bact. 34: 667-681. 1931.

Breed, R. S.
Bergey's manual of determinative bacteriology, 5th ed. Baltimore. Williams and Wilkins. 1939.

Buddingh, B. J.
A study of generalized vaccinia in chick embryos. Jour. Expt. Med. 63: 227-240. 1936.

Byerling, T. C.
A simple method for observing the chick embryo. Science. 63: 458-459. 1926.

Cary, W. E.
Virulence and toxin production in B. diphtheriae. Jour. Infect. Dis. 20: 244-246. 1917.

Cromartie, W. J.
Infection of normal and passively immunized chick embryos with Corynebacterium diphtheriae. Amer. Jour. Path. 17: 414-419. 1941.

Crowell, M. T.
Morphological and physiological variations in descendants of a single diphth ria bacilli. Jour. Bact. 11: 65-73. 1926.

Davis, L. and N. S. Ferry.
Role of the amino acids in the metabolism of Bacterium diphtheriae. Jour. Bact. 4: 217-222. 1919.

Davis, L.
Relation of bacteriological peptones to production of toxin and antitoxin. Jour. Bact. 5: 477-480. 1920.

Evans, F. E.
The action of diphtheria toxin on embryonic chicks. Jour. Immunol. 35: 398-407. 1938.

Frobisher, M., Jr.
Cystiene tellurite agar for B. diphtheriae. Jour. Infect. Dis. 60: 99-105. 1937.

Frobisher, M., Jr.
The susceptibility of chicks to the action of Corynebacterium diphtheriae and its toxin. Amer. Jour. Hyg. 33: 381-385. 1940.

Frobisher, M., E. L. Parsons and Tsun Tung.
The use of chicks in testing the virulence of B. diphtheriae. Amer. Jour. Hyg. 35: 381-384. 1942.

Goodpasture, E. W.
Vaccinal infection of the chorio-allantoic membrane of the chick embryo. Amer. Jour. Path. 8: 271-281. 1932.

Goodpasture, E. W.
The problem of infection as presented by bacterial invasion of the chorio-allantoic membrane of the chick embryo. Amer. Jour. Path. 13: 149-174. 1937.

Hadley, P. B.
Growth and toxin production on protein-free media. Jour. Infect. Dis. 3: 95-103. 1907.

Heeren, R. H.
Relation of pellicle formation and toxigenicity in diphtheria culture. Jour. Infect. Dis. 46: 161-169. 1930.

Heeren, R. H. and E. M. Magrail.
Toxin in diphtheria cultures isolated from severe cases. Jour. Infect. Dis. 46: 485-493. 1930.

Hagen, E. L. and G. Heller.
Effects of various carbohydrates on production of toxin with reference to flocculation titre. Jour. Bact. 23: 195-201. 1932.

Holt, H. D. and H. D. Wright.
The importance of the suspending fluid used in virulence tests upon Corynebacterium diphtheriae. Jour. Path. and Bact. 51: 287-296. 1940.

Kolmer, J. A.
A contribution to the bacteriology of diphtheria. Jour. Infect. Dis. 11: 56-65. 1912.

Kolmer, J. A. and E. L. Moshage.
Various methods for determining virulence. Jour. Infect. Dis. 19: 1-7. 1916.

Kolmer, J. A., S. S. Woody and E. L. Moshage.
The guinea pig test for virulence. Jour. Bact. 1: 382-396. 1916.

Kolmer, J. A. and E. L. Moshage.
Acid production by diphtheria bacilli. Jour. Infect. Dis. 19: 19-35. 1916.

Lowell, R.
The Corynebacterium pyogenes antitoxin content of animal sera. Jour. Path. and Bact. 49: 329-338. 1939.

Maver, M. E.
The growth and toxin production of Corynebacterium diphtheriae in synthetic medium. Jour. Infect. Dis. 47: 384-393. 1930.

Maver, M. E.
Chemistry of toxin produced in synthetic medium. Jour. Infect. Dis. 49: 1-12. 1931.

McGuigan, M. K. and M. Frobisher, Jr.
Mediums for study of diphtheria. Jour. Infect. Dis. 59: 22-34. 1936.

McLeod, J. W.
The different forms of Corynebacterium diphtheriae. Jour. Path. and Bact. 35: 651-652. 1932.

Mueller, T. H., K. S. Klise, E. F. Porter and A. Greybiel.
Culture requirements of diphtheria. Jour. Bact. 25: 509-519. 1933.

Povitski, O. R., M. Eisner and E. Jackson.
Effectiveness of standard diphtheria antitoxin against all types of diphtheria infection. Jour. Infect. Dis. 52: 246-252. 1933.

Shiver, W.
Diagnosis of diphtheria by means of a blood serum containing potassium tellurite. Jour. Bact. 1: 111-123. 1916.

Stark, C. N., J. M. Sherman and P. Stark.
Destruction of diphtheria toxin by bacteria. Jour. Infect. Dis. 43: 569-573. 1928.

Tsun Tung.
Experimental production and chemoprophylaxis of Corynebacterium diphtheriae in the chick embryo. Amer. Jour. Hyg. 41: 57-79. 1945.

Wadsworth, A. and M. W. Wheeler.
Attenuation and toxin production of Corynebacterium diphtheriae. Jour. Infect. Dis. 42: 179-193. 1928.

Ward, A. R.
Bacterium pyogenes and its relation to suppurative lesions in animals. Jour. Bact. 2: 619-625. 1917.

Weston, P. G. and J. A. Kolmer.
The guinea pig test for virulence. Jour. Infect. Dis. 8: 295-308. 1911.

Wilcox, H. L.
Effects of peptone on the toxigenic property of B. diphtheriae, Strain VIII. Jour. Infect. Dis. 30: 536-547. 1922.

APPENDIX

Culture Media

Loeffler's Medium

This medium as modified by the Kansas Public Health Laboratories was composed as follows: to 750 ml. of hog serum free of hemolysis and red cells was added 250 ml. of dextrose broth composed as follows:

Proteose peptone . .	15 grams
Dextrose . . .	5 grams
Meat extract . .	2.5 grams
Distilled water . .	1000 ml.

After dissolving this mixture, the pH was adjusted to 7.4. The two fuilds, the broth and the serum, were then mixed, tubed, inspissated to harden, stoppered with cork stoppers, and autoclaved at 15 pound pressure for 20 minutes.

Tellurite Medium

Various tellurite media have been proposed for isolating the organism Corynebacterium diphtheriae. Among them are the media of Shiver (1916), McLeod (1931, 1932, 1933), Frobisher (1937) to mention but a few. The one selected was a modification of the medium used by McLeod. The medium was made from a tryptose agar base composed as follows:

Tryptose . . .	20 grams
Dextrose . . .	1 gram
Sodium chloride .	5 grams
Agar Agar . . .	15 grams
Distilled water .	1000 ml.

The final pH of the medium was 6.9. This base medium was then sterilized in 250 ml. amounts at 15 pound pressure for 20 minutes. This base was then cooled to approximately 80° C. and five percent sterile sheep blood added. When completely "choclated" the media was cooled to 45° C. and then 150 ml. of a 0.045 percent solution of potassium tellurite was added. This tellurite solution was filtered through a sterile seitz filter before it was added to the medium. The entire medium was then poured into sterile petri dishes, approximately 20 ml. to a dish.

Hormone Broth

This was prepared by infusing lean veal as follows: Fresh veal free of fat was ground fine, and to each 1000 grams of veal was added 1000 milliliters of distilled water. This material was allowed to infuse over night in the ice box. This mixture was then cooked in the autoclave at 15 pound pressure for one hour. After cookin_, it was filtered through cheese cloth to remove the meat. The pH was adjusted to 7.6 by use of normal sodium hydroxide solution. This fluid was filtered through coarse filter paper, distributed in flasks and sterilized.

Fermentation base

To each liter of Hormone broth the following were added:

Proteose peptone . . .	10 grams
Sodium chloride . . .	5 grams

The pH was again adjusted to 7.6 and one milliliter of a 1.6 percent solution of brom cresol purple was added as an indicator. The broth was then sterilized in flasks and stored until used.

Addition of Carbohydrates

The three carbohydrates used were dextrose, sucrose, and starch. Dextrose and sucrose were added to the fermentation media in a final concentration of 1.0 percent. A 2.0 percent suspension of starch was made and 200 milliliters of this was added to each liter of fermentation base. Soluble starch was not used due to the possibility of partial digestion during the process of rendering soluble. These carbohydrates were then tubed approximately two and one-half milliliters to a tube. The tubes were plugged with various colored cotton to identify each carbohydrate. They were sterilized at 115° C. for ten minutes, and incubated for 48 hours to check sterility.

The medium for pellicle formation, and toxin formation and hemolysin medium was prepared by the addition of 1.0 percent proteose peptone, and 0.5 percent sodium chloride to the hormone base infusion broth. The medium was adjusted to pH 7.6, tubed or bottled, and sterilized in the autoclave at 15 pound pressure for 20 minutes.

Technique used in testing for Hemolysins

The technique used was that of Hammerschmidt, as given in Diagnostic Procedures and Reagents of the American Public Health Association (1941). Tubes of infusion media were inoculated

with the cultures under examination and incubated for 48 hours. One milliliter of this culture was then placed in a clean seriological tube and one milliliter of a 2.0 percent suspension of thrice washed human red blood cells was added. This mixture was then incubated at 37° C. for an hour and then placed in the refrigerator over night and read the next day.

On the following pages is given a chart of the culture reactions.

No.	Lab. no.	Dextrose	Sucrose	Starch	Hemolysis	Proteolysis	Pellicle	Fever-sion	Chick test	Chick cont.	Pig test	Pig cont.	Embryo 24	Embryo 48	Embryo cont.	Strain
1	Toronto	+	-	-	-	-	-	-	+	-	+	-	+	+	-	(1)
2	VII	+	-	+	+	-	+	+	+	-	+	-	+	+	-	(2)
3	XIEM	+	-	+	+	-	+	+	+	-	-	-	+	+	-	(2)
4	VIIEM	+	-	+	+	-	+	+	+	-	+	-	+	+	-	(2)
5	C.dip.Foltz	+	-	+	+	-	+	+	+	-	+	-	+	+	-	(2)
6	IA	+	+	+	-	+	+	+	-	-	-	-	+	+	+	(3)
7	IC	+	-	+	-	-	+	+	-	-	-	-	+	+	-	(2)
8	2A	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
9	2B	+	-	+	+	-	+	+	-	-	-	-	-	-	-	(2)
10	4C	+	-	-	+	-	+	-	-	-	-	-	-	-	-	(4)
11	5BA	+	+	-	-	-	+	-	-	-	-	-	-	-	-	
12	6A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
13	7	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
14	11C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(3)
15	1213	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
16	12BA	+	+	+	-	+	-	+	-	-	-	-	+	+	+	(3)
17	16A	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
18	1613	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
19	18A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(3)
20	19A	+	-	-	-	-	-	-	+	-	-	-	+	+	-	(1)
21	21C	+	-	+	-	-	+	+	-	-	-	-	-	-	-	(2)
22	21BA	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
23	23B	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
24	24B	+	+	+	-	+	+	+	+	+	+	+	+	+	+	(3)
25	24C	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
26	25B	+	-	-	-	-	-	-	+	-	-	-	+	+	-	(1)
27	26A	+	-	-	-	-	-	-	+	-	+	-	+	+	-	(1)
28	26C	+	-	-	-	-	-	-	-	-	-	-	+	+	-	(1)
29	29A	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
30	30B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
31	32B	+	-	-	-	-	-	-	+	-	-	-	+	+	-	(1)
32	32-1A	+	+	+	+	+	+	+	-	-	-	-	+	+	+	(3)
33	32-1B	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
34	32-2A	+	-	-	+	-	+	-	-	-	-	-	+	+	-	(4)
35	32-2B	+	+	+	+	+	+	+	-	-	-	-	-	-	-	(3)

No.	Lab. no.	Dex-trose	Su-crose	Starch	Hemol-ysis	Prote-olysis	Polli-cle	Rever-sion	Chick test	Chick cont.	Pig test	Pig cont.	Embryo 24	Embryo 48	Embryo cont.	Strain
36	32-2C	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
37	33A	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
38	33B	+	-	-	+	-	+	-	+	-	+	-	+	+	-	(4)
39	34A	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
40	34B	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
41	36B	+	-	-	-	-	-	-	+	-	-	-	+	+	-	(1)
42	39A	+	+	-	-	-	+	-	-	-	-	-	-	-	-	
43	42A	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
44	42B	+	-	-	-	-	-	-	-	-	-	-	+	+	-	(1)
45	43A	+	-	+	+	-	+	-	-	-	-	-	+	+	-	(2)
46	45A	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
47	45B	+	+	+	-	+	+	+	+	+	+	+	+	+	+	(3)
48	46A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(3)
49	48A	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
50	48B	+	-	-	-	-	-	-	-	-	-	-	+	+	-	(1)
51	49A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
52	49B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
53	51A	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
54	51B	+	-	+	+	-	+	+	-	-	-	-	-	-	-	(2)
55	52A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
56	52B	+	+	+	-	+	+	+	-	-	-	-	-	-	-	(3)
57	54B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
58	54C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(3)
59	56B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(3)
60	58A	+	-	+	-	-	+	+	-	-	-	-	+	+	-	(2)
61	59A	+	-	+	-	-	+	+	-	-	-	-	+	+	-	(2)
62	59B	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
63	62A	+	+	+	-	+	+	+	-	-	-	-	+	+	+	(3)
64	62B	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
65	66A	+	+	-	+	-	+	+	-	-	-	-	-	-	-	
66	71A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(3)
67	72A	+	-	+	-	-	+	+	+	-	+	-	+	+	-	(2)
68	77A	+	-	-	-	-	-	-	+	-	+	-	+	+	-	(1)
69	79A	+	+	-	-	-	+	-	-	-	-	-	-	-	-	
70	80A	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
71	80B	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)

No.	Lab. no.	Dextrose	Sucrose	Starch	Hemolysis	Proteolysis	Pellicle	Reversion	Quick test	Quick cont.	Pig test	Pig cont.	Embryo 24	Embryo 48	Embryo cont.	Strain
72	86B	+	+	+	+	+	+	+	+	+	+	+	-	-	-	(3)
73	90A	+	-	-	-	-	-	-	-	-	-	-	+	+	-	(1)
74	93A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(3)
75	93B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(3)
76	95B	+	+	-	+	-	-	-	-	-	-	-	-	-	-	
77	98B	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
78	104B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
79	105B	+	-	-	-	-	-	-	+	-	+	-	+	+	-	(1)
80	105C	+	-	+	+	-	+	+	+	-	+	-	+	+	-	(2)
81	106B	+	-	-	-	-	-	-	-	-	-	-	+	+	-	(1)
82	106A	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
83	109A	+	+	+	+	+	+	+	+	+	-	-	-	-	-	(3)
84	118A	+	-	+	-	-	+	+	-	-	-	-	+	+	-	(2)
85	120A	+	+	+	-	+	+	+	+	+	+	+	-	-	-	(3)
86	120B	+	-	-	-	-	-	-	+	-	-	-	+	+	-	(1)
87	122A	+	+	-	-	-	+	-	-	-	-	-	-	-	-	
88	129A	+	+	+	-	+	+	+	-	-	-	-	-	-	-	(3)
89	130A	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
90	131B	+	-	+	-	-	+	+	-	-	-	-	+	+	-	(2)
91	138A	+	+	-	-	-	+	-	-	-	-	-	-	-	-	
92	140	+	-	-	-	-	-	-	-	-	-	-	+	+	-	(1)
93	141A	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
94	158B	+	-	+	-	-	+	+	+	-	-	-	+	+	-	(2)
95	159A	+	+	+	-	+	+	+	+	+	+	+	+	+	+	(3)
96	161A	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
97	161B	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
98	164A	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
99	164B	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
100	164C	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
101	169A	+	-	+	+	-	+	+	+	-	-	-	+	+	-	(2)
102	170A	+	-	+	+	-	+	+	+	-	-	-	+	+	-	(2)
103	172A	+	-	+	-	-	+	+	+	-	-	-	+	+	-	(2)
104	173A	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
105	175A	+	+	+	-	+	-	+	-	-	-	-	-	-	-	(3)
106	178A	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
107	180A	+	-	+	+	-	+	+	-	-	-	-	-	-	-	(2)

No.	Lab. no.	Dextrose	Sucrose	Starch	Hemolysis	Proteolysis	Fellicle	Fever	Chick test	Chick cont.	Pig test	Pig cont.	Embryo 24	Embryo 48	Embryo cont.	Strain
108	184B	+	+	-	-	-	+	-	-	-	-	-	-	-	-	
109	185NA	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
110	188A	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
111	188B	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
112	192A	+	-	-	-	-	-	-	-	-	+	-	+	+	-	(1)
113	201A	+	+	+	-	+	+	+	-	-	-	-	-	-	-	(3)
114	206A	+	+	-	-	-	+	-	-	-	-	-	-	-	-	
115	206B	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
116	206C	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
117	207B	+	+	+	-	+	+	+	+	+	-	-	+	+	+	(3)
118	208A	+	+	-	-	-	+	-	-	-	-	-	-	-	-	
119	208B	+	-	-	-	-	-	-	-	-	-	-	+	+	-	(1)
120	209B	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
121	210A	+	-	-	-	-	-	-	+	-	-	-	+	+	-	(1)
122	211A	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
123	211B	+	-	-	-	-	-	-	-	-	-	-	+	+	-	(1)
124	212A	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
125	212B	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
126	229A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(3)
127	230A	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
128	232A	+	+	-	-	-	+	-	-	-	-	-	-	-	-	
129	234A	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
130	236A	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
131	239A	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
132	241A	+	-	+	-	-	+	+	-	-	-	-	-	-	-	(2)
133	243A	+	+	+	+	+	+	+	-	-	-	-	-	-	-	(3)
134	246A	+	-	+	+	-	+	+	+	-	-	-	+	+	-	(2)
135	246B	+	-	+	-	-	+	+	+	-	-	-	+	+	-	(2)
136	270A	+	-	-	-	-	-	-	-	-	-	-	+	+	-	(1)
137	271E	+	+	-	-	-	+	-	-	-	-	-	-	-	-	
138	287	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
139	295B	+	-	+	-	-	+	+	-	-	-	-	-	-	-	(2)
140	297B	+	+	+	-	+	+	+	+	+	+	+	+	+	+	(3)
141	302A	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
142	302B	+	-	+	-	-	+	+	+	-	+	-	+	+	-	(2)

No.	Lab. no.	Dex-trose	Su-crose	Starch	Hemol-ysis	Prote-olysis	Pelli-cle	Rever-sion	Chick test	Chick cont.	Pig test	Pig cont.	Embryo 24	Embryo 48	Embryo cont.	Strain
143	304A	+	+	+	+	+	+	+	-	-	-	-	-	-	-	(3)
144	306A	+	-	+	+	-	+	+	+	-	-	-	+	+	-	(2)
145	306B	+	+	+	+	+	+	+	+	+	-	-	-	-	-	(3)
146	306C	+	-	-	-	-	-	-	-	-	-	-	+	+	-	(1)
147	308A	+	-	+	-	-	+	+	+	-	-	-	+	+	-	(2)
148	314E	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
149	315B	+	-	-	-	-	-	-	-	-	-	-	+	+	-	(1)
150	320A	+	+	+	-	+	+	+	+	+	+	+	-	-	-	(3)
151	320B	+	+	+	-	+	+	-	-	-	-	-	-	-	-	(3)
152	321A	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
153	322A	+	-	+	+	-	+	+	+	-	-	-	+	+	-	(2)
154	322B	+	-	+	+	-	+	+	+	-	-	-	+	+	-	(2)
155	324A	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
156	324B	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
157	326A	+	+	-	-	-	+	-	-	-	-	-	-	-	-	
158	327B	+	-	-	-	-	-	-	-	-	-	-	+	+	-	(1)
159	328A	+	+	+	-	+	+	+	+	+	-	-	-	-	-	(3)
160	331A	+	-	-	+	-	+	-	-	-	-	-	+	+	-	(4)
161	331B	+	-	-	-	-	-	-	+	-	+	-	+	+	-	(1)
162	332A	+	+	+	+	+	+	+	+	+	+	+	-	-	-	(3)
163	332B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(3)
164	336B	+	+	+	-	+	+	+	+	+	+	+	+	+	+	(3)
165	339B	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
166	340A	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
167	344A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(3)
168	346A	+	-	+	-	-	+	-	-	-	-	-	+	+	-	(2)
169	357B	+	-	-	+	-	+	-	+	-	-	-	+	+	-	(4)
170	359A	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
171	359B	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
172	374A	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
173	379A	+	+	+	-	+	+	+	+	+	+	+	+	+	+	(3)
174	381A	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
175	381A	+	-	-	-	-	-	-	-	-	-	-	+	+	-	(1)
176	391B	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
177	392A	+	-	-	+	-	+	-	-	-	-	-	+	+	-	(4)
178	392B	+	-	-	+	-	+	-	-	-	-	-	+	+	-	(4)

No.	Lab. no.	Dex-trose	Su-crose	Starch	Hemol-ysis	Prote-ase	Pelli-cle	Rever-sion	Chick test	Chick cont.	Pig test	Pig cont.	Embryo 24:48	Embryo cont.	Strain
179	396A	-	-	-	-	-	-	-	-	-	-	-	-	-	
180	405TB	+	-	-	-	-	-	-	-	-	-	-	-	-	(1)
181	405TC	+	-	-	-	-	-	-	-	-	-	-	-	-	(1)
182	410A	+	+	-	-	-	-	-	-	-	-	-	-	-	
183	413B	+	-	-	-	-	-	-	-	-	-	-	-	-	(1)
184	414A	+	-	-	+	-	+	-	+	-	-	-	+	-	(4)
185	414B	+	-	+	+	-	+	-	+	-	-	-	+	-	(2)
186	414C	+	-	-	-	-	-	-	-	-	-	-	-	-	(1)
187	418B	+	+	-	-	-	+	-	-	-	-	-	-	-	
188	421A	+	-	-	+	-	+	-	+	-	+	-	+	-	(4)
189	422A	+	+	-	-	-	-	-	-	-	-	-	-	-	
190	423A	-	-	-	-	-	+	-	-	-	-	-	-	-	
191	427A	+	-	-	-	-	-	-	-	-	-	-	+	-	(1)
192	427B	+	+	+	-	+	+	+	+	+	-	-	+	+	(3)
193	428A	+	-	-	+	-	+	-	-	-	-	-	+	-	(4)
194	428B	+	-	+	-	-	+	+	-	-	-	-	+	-	(2)
195	429A	-	-	-	-	-	+	-	-	-	-	-	-	-	
196	432A	+	+	+	-	+	+	+	-	-	-	-	-	-	(3)
197	432B	+	+	+	-	+	+	+	-	-	-	-	+	+	(3)
198	436A	+	-	-	-	-	-	-	-	-	-	-	-	-	(1)
199	436B	+	-	-	-	-	-	-	-	-	-	-	+	-	(1)
200	439A	+	-	+	+	-	+	+	+	-	-	-	+	-	(2)
201	442A	+	+	+	-	+	+	+	+	+	+	+	+	+	(3)
202	442B	+	+	+	+	+	+	+	+	+	+	+	+	+	(3)
203	448A	-	-	-	-	-	+	-	-	-	-	-	-	-	
204	493A	+	-	-	-	-	-	-	-	-	-	-	-	-	(1)
205	494A	+	+	+	-	+	+	+	+	+	+	+	+	+	(3)
206	495A	-	-	-	-	-	+	-	-	-	-	-	-	-	
207	498A	+	-	-	-	-	-	-	-	-	-	-	+	-	(1)
208	501A	+	-	-	-	-	-	-	-	-	-	-	+	-	(1)
209	506A	+	-	+	+	-	+	+	+	-	-	-	+	-	(2)
210	508A	+	+	-	-	-	+	-	-	-	-	-	-	-	
211	527A	+	-	-	+	-	+	-	-	-	-	-	-	-	(4)
212	529A	+	-	-	-	-	-	-	+	-	-	-	+	-	(1)
213	529B	+	-	-	-	-	-	-	+	-	-	-	+	-	(1)
214	531A	+	-	-	-	-	-	-	+	-	-	-	+	-	(1)
215	531B	+	+	+	+	+	+	+	+	+	+	+	+	+	(3)

No.	L.D. no.	Dex-trose	Su-crose	Starch	Hemol-ysis	Prote-olysis	Polli-cle	Rever-sion	Chick test	Chick cont.	Pig test	Pig cont.	Embryo 24	Embryo 48	Embryo cont.	Strain
216	547A	+	-	-	-	-	-	-	+	-	-	-	+	+	-	(1)
217	547B	+	-	-	-	-	-	-	+	-	-	-	+	+	-	(1)
218	576	+	-	-	+	-	+	-	-	-	-	-	+	+	-	(4)
219	577	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
220	580	+	-	-	-	-	-	-	+	-	+	-	+	+	-	(1)
221	582	+	-	-	+	-	+	-	+	-	+	-	+	+	-	(4)
222	584	+	-	-	-	-	-	-	+	-	-	-	+	+	-	(1)
223	586	+	+	+	-	+	-	+	+	+	+	+	+	+	+	(3)
224	591	+	-	+	-	-	+	+	+	-	+	-	+	+	-	(2)
225	593	+	-	-	-	-	-	-	-	-	-	-	+	+	-	(1)
226	594A	+	-	-	-	-	-	-	-	-	-	-	+	+	-	(1)
227	594B	+	-	+	-	-	+	+	-	-	-	-	+	+	-	(1)
228	594E	+	+	+	+	+	+	+	-	-	-	-	+	+	+	(3)
229	596	+	+	+	+	+	+	+	+	+	+	+	-	-	-	(3)
230	599E	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
231	649	+	-	-	-	-	-	-	+	-	+	-	+	+	-	(1)
232	651	+	-	-	-	-	-	-	+	-	+	-	+	+	-	(1)
233	652A	+	+	+	+	+	+	+	-	-	-	-	-	-	-	(3)
234	652B	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
235	654	+	-	-	-	-	-	-	+	-	-	-	+	+	-	(1)
236	658	-	-	-	-	-	-	+	-	-	-	-	-	-	-	
237	661	+	-	+	+	-	+	+	-	-	-	-	+	+	-	(2)
238	662	+	+	+	+	+	+	+	+	+	+	+	-	-	-	(3)
239	663	+	+	+	+	+	+	+	-	-	-	-	-	-	-	(3)
240	669	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
241	671	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
242	674	+	-	-	-	-	-	-	-	-	-	-	+	+	-	(1)
243	676	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
244	677	+	-	-	-	-	-	-	+	-	-	-	+	+	-	(1)
245	688B	+	-	-	+	-	+	-	-	-	-	-	+	+	-	(4)
246	682	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
247	684	+	+	-	-	-	+	-	-	-	-	-	-	-	-	
248	694	+	-	-	-	-	-	-	-	-	-	-	+	+	-	(1)
249	697	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
250	698	+	+	-	-	-	+	-	-	-	-	-	-	-	-	

No.	Lab. no.	Dextrose	Sucrose	Starch	Hemolysis	Proteolysis	Gelatin	Reversion	Chick test	Chick cont.	Pig test	Pig cont.	Embryo 24	Embryo 48	Embryo cont.	Strain
251	700	+	-	-	-	-	-	-	+	-	-	-	+	+	-	(1)
252	701	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
253	705	+	-	-	-	-	-	-	+	-	-	-	+	+	-	(1)
254	713	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
255	718	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
256	719	+	+	+	-	+	+	+	-	-	-	-	+	+	+	(3)
257	720	+	-	-	-	-	-	-	+	-	+	-	+	+	-	(1)
258	722	+	+	+	+	+	+	+	+	+	+	+	-	-	-	(3)
259	726	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(3)
260	728A	+	-	+	+	-	+	+	-	-	-	-	+	+	-	(2)
261	728B	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
262	730	+	+	-	-	-	+	-	-	-	-	-	-	-	-	
263	731	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
264	733	+	-	-	-	-	-	-	+	-	-	-	+	+	-	(1)
265	735A	+	-	-	+	-	+	-	-	-	-	-	-	-	-	(4)
266	735B	+	+	-	-	-	+	-	-	-	-	-	-	-	-	
267	736	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
268	737	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
269	739	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
270	744	+	+	+	+	+	+	+	-	-	-	-	-	-	-	(3)
271	746	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
272	748	+	-	-	-	-	-	-	-	-	-	-	+	+	-	(1)
273	752	+	+	+	+	+	+	+	-	-	-	-	-	-	-	(3)
274	754	+	+	-	-	-	+	-	-	-	-	-	-	-	-	
275	756	+	-	-	+	-	+	-	-	-	-	-	-	-	-	(4)
276	758	+	-	-	+	-	+	-	-	-	-	-	-	-	-	(4)
277	759	+	+	-	-	-	+	-	-	-	-	-	-	-	-	
278	763	+	-	-	-	-	-	-	+	-	+	-	+	+	-	(1)
279	767	+	-	-	-	-	-	-	-	-	-	-	+	+	-	(1)
280	769A	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
281	769B	+	+	+	+	+	+	+	-	-	-	-	-	-	-	(3)
282	779	+	-	+	+	-	+	+	+	-	+	-	+	+	-	(2)
283	785A	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
284	785B	+	+	-	-	-	+	-	-	-	-	-	-	-	-	
285	787	+	+	-	-	-	+	-	-	-	-	-	-	-	-	
286	789	-	-	-	-	-	+	-	-	-	-	-	-	-	-	

No.	Lab. no.	Dextrose	Sucrose	Starch	Hemolysis	Proteolysis	Gelatinase	Reversion	Chick test	Chick cont.	Pig test	Pig cont.	Embryo 24	Embryo 48	Embryo cont.	Strain
287	790	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
288	791	+	-	-	-	-	-	-	-	-	-	-	+	+	-	(1)
289	794	+	-	-	-	-	-	-	-	-	-	-	+	+	-	(1)
290	797	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
291	803	+	+	-	-	-	+	-	-	-	-	-	-	-	-	
292	804A	+	+	+	+	+	+	+	-	-	-	-	+	+	+	(3)
293	804B	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
294	806	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
295	830	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
296	837A	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
297	837B	+	-	-	+	-	+	+	+	-	+	-	+	+	-	(4)
298	837C	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
299	853A	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
300	853B	+	+	+	+	+	+	+	+	+	+	+	-	-	-	(3)
301	857	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(3)
302	859A	+	+	+	+	+	+	+	+	+	-	-	+	+	+	(3)
303	859B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(3)
304	867	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
305	867A	+	+	-	-	-	+	-	-	-	-	-	-	-	-	
306	868	+	-	+	-	-	+	+	-	-	-	-	-	-	-	(2)
307	869T	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
308	870	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
309	871A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(3)
310	871B	+	+	+	+	+	+	+	+	+	-	-	+	+	+	(3)
311	872	+	+	+	+	+	+	+	+	+	+	+	-	-	-	(3)
312	877	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
313	880	+	-	+	+	-	+	+	-	-	-	-	+	+	-	(2)
314	881	+	+	+	+	+	+	+	+	+	+	+	-	-	-	(3)
315	882	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
316	884	+	+	+	+	+	+	+	+	+	+	+	-	-	-	(3)
317	885	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
318	886	+	+	+	+	+	+	+	+	+	+	+	-	-	-	(3)
319	888E	+	-	-	-	-	-	-	-	-	-	-	+	+	-	(1)
320	928TB	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
321	929B	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
322	968A	+	+	+	+	+	+	+	-	-	-	-	-	-	-	(3)

No.	Lab. no.	Dex-trose	Su-crose	Starch	Hemol-ysis	Prote-olysis	Pelli-cle	Never-sion	Chick test	Chick cont.	Pig test	Pig cont.	Embryo 24	Embryo 48	Embryo cont.	Strain
323	968B	+	+	-	-	-	+	-	-	-	-	-	-	-	-	
324	976	+	-	-	-	-	-	-	-	-	-	-	+	+	-	(1)
325	978E	+	-	+	+	-	+	+	+	-	+	-	+	+	-	(2)
326	984	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(3)
327	987	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
328	988B	+	-	+	-	+	+	+	+	-	-	-	+	+	-	(2)
329	988N	+	-	+	-	+	+	+	+	-	-	-	+	+	-	(2)
330	988T	+	-	+	-	+	+	+	+	-	-	-	+	+	-	(2)
331	995	+	-	-	-	-	-	-	+	-	-	-	+	+	-	(1)
332	1012N	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
333	1012T	+	+	+	+	+	+	+	-	-	-	-	+	+	+	(3)
334	1013N	+	-	+	-	-	+	+	+	-	+	-	+	+	-	(2)
335	1013T	+	+	+	+	+	+	+	-	-	-	-	+	+	+	(3)
336	1016N	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
337	1016T	+	-	-	-	-	-	-	-	-	-	-	+	+	-	(1)
338	1018B	+	+	+	+	+	+	+	+	+	+	+	-	-	-	(3)
339	1018C	+	-	+	+	-	+	+	-	-	-	-	+	+	-	(2)
340	1045N	+	+	+	+	+	+	+	-	-	-	-	-	-	-	(3)
341	1045T	+	+	+	+	+	+	+	-	-	-	-	-	-	-	(3)
342	1047	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
343	1051	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
344	1062	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(3)
345	1068T	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
346	1068N	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
347	1076	+	+	+	+	+	+	+	-	-	-	-	-	-	-	(3)
348	1091	+	-	-	-	-	-	-	+	-	-	-	+	+	-	(1)
349	1092	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
350	1094	+	+	+	+	+	+	+	-	-	-	-	-	-	-	(3)
351	1108	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(3)
352	1129	+	-	-	-	-	-	-	-	-	-	-	+	+	-	(1)
353	1147	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
354	1155	+	+	+	+	+	+	+	+	+	+	+	-	-	-	(3)
355	1160	+	-	+	+	-	+	+	-	-	-	-	+	+	-	(2)
356	1172	+	+	-	-	-	+	-	-	-	-	-	-	-	-	
357	1278T	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(3)
358	1279A	+	-	+	+	-	+	+	+	-	+	-	+	+	-	(2)

No.	Lab. no.	Dex-trose	Su-crose	Starch	Hemol-ysis	Prote-olysis	Pelli-cle	Fever-sion	Chick test	Chick cont.	Pig test	Pig cont.	Embryo 24	Embryo 48	Embryo cont.	Strain
359	1279B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(3)
360	1285	+	-	-	+	-	+	+	-	-	-	-	-	-	-	(4)
361	1295T	+	+	+	+	+	+	+	-	-	-	-	-	-	-	(3)
362	1297N	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(3)
363	1314	+	-	+	+	-	+	+	+	-	+	-	+	+	-	(2)
364	1377T	+	+	+	+	+	+	+	+	+	+	+	-	-	-	(3)
365	1379	+	+	+	+	+	+	+	+	+	+	+	-	-	-	(3)
366	1396	+	-	-	-	-	-	-	-	-	-	-	+	+	-	(1)
367	1398	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
368	1401	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
369	1404	+	-	-	-	-	-	-	+	-	-	-	+	+	-	(1)
370	1409A	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
371	1409B	+	-	+	-	-	+	+	-	-	-	-	+	+	-	(2)
372	1409C	+	-	+	-	-	+	+	-	-	-	-	+	+	-	(2)
373	1409D	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
374	1409E	+	-	+	+	-	+	+	-	-	-	-	+	+	-	(2)
375	1421	+	-	+	+	-	+	+	+	-	-	-	+	+	-	(2)
376	1436T	+	+	-	-	-	+	-	-	-	-	-	-	-	-	
377	1436B	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
378	1445	+	-	-	-	-	-	-	-	-	-	-	-	-	-	(1)
379	1447E	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
380	1454	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(3)
381	1571A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(3)
382	1704	+	-	-	+	-	+	+	+	-	+	-	+	+	-	(4)
383	1018A	+	-	+	+	-	+	+	-	-	-	-	+	+	-	(2)
384	1706	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(3)
385	1707B	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
386	1707C	+	-	-	-	-	-	-	-	-	-	-	+	+	-	(1)
387	1709	+	-	-	+	-	+	-	-	-	-	-	-	-	-	(4)

(1) Corynebacterium diphtheriae intermedius.
(2) Corynebacterium diphtheriae gravis.
(3) Corynebacterium pyogenes.
(4) Corynebacterium diphtheriae mitis.